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Original Article

Characterization of Hum j 6, a Major Allergen From *Humulus japonicus* Pollen, the Primary Cause of Weed Pollinosis in East Asia

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ABSTRACT

Purpose: *Humulus japonicus* (*HJ*) is one of the most important causes of weed pollinosis in East Asia. The 10 kDa protein with pI 10 in 2-dimensional gel has been recognized as the representative major allergen of *HJ*, but its major allergens have not been characterized. This study aimed to characterize the major allergen of *HJ*.
Methods: A major allergen in Japanese hop was detected by proteome analysis; it was purified to homogeneity and its sequence was obtained by transcriptome analysis. The recombinant proteins were produced in *Escherichia coli* and *Pichia* expression systems, and their immunoglobulin E (IgE) reactivities were compared to those of the natural counterpart. We also analyzed post-translational modifications such as glycosylation and phosphorylation.
Results: Pectin methylesterase inhibitor, Hum j 6, was found to be the major allergen of *HJ*, and *in silico* signal peptide prediction corresponds to a 15.1 kDa protein with a theoretical pI of 8.28. Natural Hum j 6 was recognized by IgE antibodies from 86.4% (19/22) of *HJ* pollinosis patients, whereas the recombinant proteins did not show strong IgE reactivity. No glycosylation was detected, while at least 15 phosphorylated amino acids, possibly causing the pI and molecular weight shift, were detected by tandem mass spectrometry analysis.
Conclusions: Hum j 6 was identified as the representative major allergen of *HJ* and seems to be modified significantly after translation. These findings are useful for the development of component-resolved diagnosis and immunotherapy.

Keywords: Pollinosis; diagnosis; immunotherapy; allergens; glycosylation; phosphorylation

INTRODUCTION

Humulus japonicus (*HJ*), also known as *Humulus scandens* or Japanese hop, is the primary cause of weed pollinosis in East Asia. Moreover, it is now considered an invasive species in North America and already established in Europe.[1] *HJ* shows no cross-reactivity with common hop, a closely related species,[2] or other important allergenic weeds.[3] Several proteins belonging to allergenic families, such as pathogenesis-related 1 (PR-1) protein, pectin methylesterase (PME), polygalacturonase, and profilin, were identified in *HJ* pollen by expressed sequence tag analysis, but displayed low allergenicity.[4,5]

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Disclosure
There are no financial or other issues that might lead to conflict of interest.

A previous study identified a 10 kDa component as the representative major allergen of *HJ* pollinosis.[3] However, the molecular details of this major allergen have not been characterized. Identification of this allergen proved to be difficult, as the corresponding spot is often missing in 2-dimensional (2D) gel analysis. Moreover, the lack of database entries regarding *HJ* made identification of this component using mass spectrometry (MS) difficult.

Identification and characterization of major allergenic molecules is important to standardize and improve the quality of allergen extracts.[6] Great advances have been made in characterizing allergen properties using molecular biology techniques, and many efforts are being made to replace the extracts with recombinant molecules of major allergen.[7,8] So far, molecular cloning and allergenic characterization of the major allergen from *HJ* pollen has not been successful.

In this study, we purified the major allergen from *HJ* and characterized its properties. This allergen was designated as Hum j 6 according to the World Health Organization/International Union of Immunological Societies Allergen nomenclature sub-committee.[9]

MATERIALS AND METHODS

Patients and serum samples

Skin prick tests or ImmunoCAP (Thermo Fisher Scientific, Waltham, MA, USA) were routinely performed on patients who were referred to the Allergy-Asthma Center at Severance Hospital, Seoul, Korea. Among the tested patients, 22 sensitized to *HJ* with typical weed pollinosis symptoms (age range 12–70 years, mean 43.5 years; 8 males and 14 females) were enrolled in this study. Typical symptoms were seasonal allergic rhinoconjunctivitis from mid-August to early October. Another 22 healthy individuals negative for *HJ* sensitivity and without symptoms of allergic rhinoconjunctivitis (age range 3–25 years, mean 14 years; 9 males and 13 females) were included as negative controls (**Table**). Serum samples were collected and kept at −20°C until use. This study was approved by the Institutional Review Board of Yonsei University College of Medicine (4-2017-1197). All patients completed the written consent form to participate in the study before collection of blood.

Proteomic analysis of the allergen

Allergen extract was desalted using a Zeba spin desalting column (7 kDa cutoff; Thermo Fisher Scientific) and rehydrated in 7 M urea, 2 M thiourea, 4% CHAPS, 2.5% dithiothreitol. The rehydrated sample was loaded (70 μL) onto 7 cm of H 6–11 strip, and isoelectric focusing was carried out (28,500 V/hr). Subsequently, proteins were separated on an 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie Brilliant Blue G250 or electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm; Millipore, Burlington, MA, USA).

Immunoglobulin E (IgE) reactive spot was probed with alkaline phosphatase-conjugated goat anti-human IgE (epsilon chain specific; Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. The color was developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega, Madison, WI, USA).

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Table. Clinical features of enrolled subjects

No.	Sex	Age	Diagnosis/ Symptom	Sensitization profile*	IgE (kU_A/L) to Japanese hop (w22)	Total IgE (kU/L)
1	F	59	AR	d2, e1, e2, g6, t2, t3, t5, t7, t10, w22	4.51	181
2	M	46	AR	f95, g1, g7, t12, g2, g3, g6, i6, t7, w1, w6, w8, w11, w14, w22	5.76	56.8
3	M	23	AR, AC	m1, t1, t2, t3, t5, t7, t8, t10, t11, t70, w1, w5, w7, w8, w10, w12, w22	32	83.1
4	M	56	AR, AS	f14, t1, t2, t3, t5, t7, t15, t70, w5, w7, w12, w22	20.6	306
5	F	45	AR	d1, d2, t3, t7, w1, w5, w7, w10, w16, w22	17.5	ND
6	F	64	AR	i6, w22	6.06	30
7	M	58	AR	w22	11.8	ND
8	F	49	AR, AC	w1, w6, w10, w22	> 100	232
9	F	41	AR, AC	w1, w6, w8, w12, w22	30.1	230
10	M	32	AR, EB	d1, d2, d72, e1, g2, g3, g5, g6, g8, g12, m3, m5, t1, t2, t3, t4, t7, t8, t10, t11, t12, t15, t16, t70, w1, w5, w7, w9, w10, w12, w22	15.8	2,070
11	M	49	AR, AC	w1, w22	31.1	ND
12	F	64	AR, AC	d1, d2, w1, w22	2.55	183
13	M	18	AR	t7, w22	33.3	ND
14	F	12	AR	d2, e5, w22	229.7	ND
15	F	54	AR	d1, d2, w1, w6, w22	> 100	356
16	F	30	AR, AD	d1, d2, d72, g5, g6, m1, m2, m3, m5, t10, t19, t70, w5, w10, w12, w22	> 100	ND
17	F	25	AR, AC	d2, e2, g5, t1, t8, t19, w1, w5, w7, w8, w12, w22	12.7	ND
18	F	56	AR	w6, w22	34.7	ND
19	M	58	AR	t2, t3, t7, t15, w5, w7, w12, w22	16.9	ND
20	F	70	AR	w22	33.1	108
21	F	23	AS	d1, d2, w1, w9	4.56	86.5
22	F	26	AR	d1, d2, e2, m6, w5, w7, w9, w22	13.5	165

IgE, immunoglobulin E; AC, allergic conjunctivitis; AD, atopic dermatitis; AR, allergic rhinitis; AS, allergic asthma; EB, eosinophilic bronchitis; ND, not determined.
*d2, *Dermatophagoides farinae*; e1, Cat dander; e2, Dog hair; e5, Dog dander; f95, Peach; g5, Rye-grass; g6, Timothy grass; i6, German cockroach; 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; t15, White ash; w1, Common ragweed; w5, Wormwood; w6, Mugwort; w8, Dandelion;; w22, Japanese hop.

Purification of the major allergen

HJ pollen extract was obtained from Prolagen Ltd. (Seoul, Korea). Natural PME inhibitor (PMEI), designated Hum j 6, was purified by 3 steps of chromatography. First, cation exchange chromatography using CM52 cellulose (GE Healthcare Bioscience, Little Chalfont, UK) was carried out in 5 mM sodium acetate, pH 5.2. Protein was eluted with a linear gradient of 0.5 M NaCl. Next, the sample was purified by strong cation exchange chromatography using a 1-mL Mono S column (GE Healthcare Bioscience). The protein was dialyzed in 50 mM Tris-Cl, pH 8.0. Elution was done with a linear gradient of 1 M NaCl. Finally, gel filtration chromatography was performed in phosphate-buffered saline (PBS), pH 7.4 using a Sephadex G75 16/60 column (GE Healthcare Bioscience). The purified protein was subjected to Edman degradation using Procise 491 HT protein sequencer (Applied Biosystems, Waltham, MA, USA) at the Korea Basic Science Institute after transfer onto PVDF membrane (0.45 μm; Millipore).

Cloning and polymorphism analysis

Total RNA was extracted, and mRNA library was prepared and sequences were analyzed as described previously.[10] RNAseq contigs homologous to PMEI sequences as determined by Edman degradation were analyzed to identify a clone encoding Hum j 6.

Reverse transcription polymerase chain reaction (PCR) was performed to investigate the polymorphism and identify relevant isoforms. cDNA was synthesized using total RNA as a template and PCR reaction was performed using ExTaq DNA polymerase with proofreading activity (forward primer; 5'-ATGGATTTGATCTCTAGCACATG-3' and reverse primer; 5'-TCAACTGAGTTGGTTTGTGATGG-3'). The PCR-amplified product was cloned into a pCR4-TOPO vector (Invitrogen, Waltham, MA, USA) and DNA sequences were determined at BioFact (Daejeon, Korea).

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3D structure modeling and multiple sequence alignment

The 3D structure of mature Hum j 6 was modeled by the automated comparative protein modeling server Swiss model using kiwi PMEI (PDB: 1XG2.1B) structure as template. UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) was used for graphical representation and to highlight details of the model. Multiple sequence alignment of Hum j 6 isoforms and PMEI allergens were performed with Clustal O program.

Production of recombinant allergens

For the production of recombinant protein in an *Escherichia coli* system, the ORF encoding the mature sequence of the major isoform was amplified by PCR (forward primer 5'-TAAGGCCTCTGTCGACGATTTGATCTCTAGCACATG-3' and reverse primer 5'-CAGAATTCGCAAGCTTACTGAGTTGGTTTGTGATGG-3'. The PCR product was ligated into a pET6xHN-C vector (Clontech Laboratories, Inc., a Takara Bio Company, Kusatsu, Japan) by the InFusion cloning system (Clontech Laboratories, Inc.). DNA sequences of cloned plasmids were determined in both directions. A total of 54 plasmid clones were analyzed. Recombinant Hum j 6 was expressed in *E. coli* Origami™ 2 (DE3) pLysS (Novagen), which allows disulfide bond formation, and purified both from soluble fraction and inclusion bodies.

Recombinant Hum j 6 expressed in *Pichia* was obtained as described previously with slight modifications.[11] DNA was synthesized with a mutation of the internal *Xho* I restriction site (CTCGAG to CAGTAG) without a change of amino acids in the original sequence containing a signal cleavage site (GAGGCTGAGCT) and *Bam* HI and *Not* I sites for the expression of Hum j 6 in *Pichia* system (5'-CTCGAGAAAAGAGAGGCT-3' at -5' and 5'-CATCATCATCATCATCATGCGGCCGC-3' at -3'). A His tag was also added at the C-terminus of the protein. The insert was removed and then ligated into a pPIC9 vector; the plasmid linearized with *Sal* I was transformed into *Pichia pastoris* GS115 strain. Recombinant Hum j 6 was purified from the culture supernatant after 2 days of culturing. Proteins were precipitated with 50% to 75% NH_4SO_4 and dialyzed for purification. Recombinant proteins expressed both in *E. coli* and *Pichia* were purified using Ni-affinity chromatography.

A mutant, C134A, with a sequence of GCC instead of TGC was synthesized in pBT7-N-His expression vector (Bioneer, Daejeon, Korea) in order to prevent disulfide bridge formation to Cys at 134th position. The expressed C134A should have N-terminal tagging sequence (MHHHHHHSSGLVPRGSEFSQQDSD-) derived from vector. It was expressed in *E. coli* Origami™ 2 (DE3) pLysS (Novagen) and purified from inclusion bodies using Ni-affinity chromatography.

Analysis of post-translational modifications

To identify potential post-translational modifications, glycoprotein staining was carried out using a Glycoprotein Staining kit (Pierce™). Horseradish peroxidase and soybean trypsin inhibitor provided in the kit were used as positive and negative controls. A change in IgE reactivity to the purified natural protein was also evaluated after treatment with calf intestinal phosphatase (CIP; Thermo Fisher Scientific) using immunoblot analysis. For dephosphorylation of Hum j 6, 2 μg of natural Hum j 6 was treated with 10 U of CIP at 37°C for 1 hour.

Liquid chromatography-coupled electrospray ionization MS/MS was performed at ProteomeTech (Seoul, Korea) to identify potential phosphorylation sites by examining a mass increment relative to the deduced amino acid sequence.

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Enzyme-linked immunosorbent assay (ELISA) and IgE-immunoblotting inhibition assays

IgE reactivity against purified proteins was assessed by ELISA. Purified proteins (2 μg/mL) were coated onto a microplate overnight in 0.05 M carbonate buffer, pH 9.6. After blocking with 3% skim milk in PBS containing 0.05% Tween 20, serum samples diluted 1:4 in PBS containing 1% bovine serum albumin (BSA) were added and plates were incubated for 1 hour. IgE antibodies were detected by biotinylated goat anti-human IgE (1:1,000) (Vector, Burlingame, CA, USA) and streptavidin-peroxidase conjugate (1:1,000) (Sigma-Aldrich). The color was developed by adding the substrate 3,3',5,5'-tetramethyl-benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). After stopping the enzyme reaction with 0.5 M H_2SO_4, absorbance at 450 nm was measured. The mean absorbance plus 2 standard deviations of the sera from healthy controls was used as a cut-off value.

The purified natural Hum j 6 and pollen extract were evaluated by SDS-PAGE under reducing conditions and then electro-transferred to a PVDF membrane. Subsequently, IgE reaction to the membrane blotted with natural Hum j 6, or the extract was stained with Coomassie blue. Specific IgE binding proteins were probed with pooled sera prepared from 5 sera samples showing high specific IgE titers against natural Hum j 6 (serum 3, 5, 8, 17, 22).

For the inhibition assay of natural Hum j 6, pooled serum was preincubated overnight at 4°C with 20 μg/mL natural Hum j 6 as an inhibitor. As a negative control, 1% BSA was used. The IgE reaction of preincubated sera with the PVDF membrane blotted with the extract was analyzed as described above. The membranes were probed with the alkaline phosphatase–conjugated goat anti-human IgE (Sigma-Aldrich) at 1:1,000 dilution and NBT/BCIP (Promega).

RESULTS

Purification and identification of Hum j 6, a PMEI

An IgE reactive spot was detected around 10 kDa with a high pI (about 10) using 2D immunoblot analysis (**Fig. 1**). The protein was further purified by anion exchange chromatography and then subjected to Edman degradation and tandem MS analysis. The N-terminal sequence determined by Edman degradation was DLISSTCSKALYKDLCEKTL. Notably, no significant match was found by tandem MS analysis. Sometimes partially degraded proteins were observed during purification. However, N-terminal sequence was the same as the intact protein determined by Edman degradation, implying a degradation at the C-terminus. The identified N-terminal sequence showed a significant match with the PME/invertase inhibitor protein family.

Analysis of the primary sequence and detection of post-translational modifications

A single sequence corresponding to Hum j 6 was obtained by transcriptome analysis of *HJ* pollen. The full-length sequence including a signal peptide encodes a 17.789 kDa protein with a pI value of 7.28. Mature Hum j 6 as identified by Edman degradation and *in silico* signal peptide prediction corresponds to a 15.112 kDa protein with a theoretical pI of 8.28. Notably, Hum j 6 is often detected as a 10 kDa protein with pI 10 in gel analysis, implying some modification after translation or unusual gel migration behavior. In polymorphism analysis, sequences of 54 clones revealed 11 Hum j 6 variants (**Fig. 2**). However, only 2 variants seem to be important, considering the possible sequencing error for the other 9 variants that showed only sporadic

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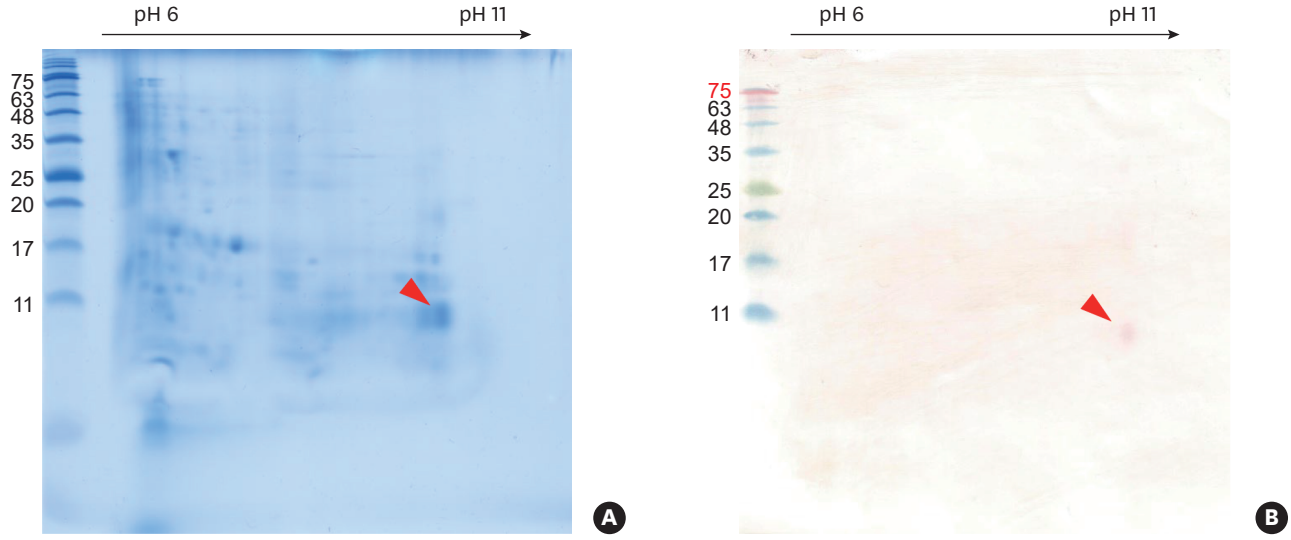

Fig. 1. Identification of the major allergen from *Humulus japonicus* pollen. Two-dimensional gel analysis (A) and immunoglobulin E immunoblotting (B) were performed to identify the allergen. The allergen was purified to homogeneity by ion-exchange chromatography.

single amino acid substitutions at different positions. The 2 major variants accounting for 96.3% of sequences have only one amino acid substitution at the 61st residue from glycine (59.26%; GenBank Accession No. ON399181) to alanine (24.07%; ON399182). The recombinant proteins were produced based on the major isoform with glycine at the 61st residue.

The N-terminal sequence of the purified protein suggests that 24 amino acids at the N-terminus are cleaved during protein maturation. Therefore, mature Hum j 6 represents a 15.112 kDa protein. Interestingly, the calculated isoelectric point of the mature protein was 8.28 whereas that of purified Hum j 6 was approximately 10.0. This difference might be due to post-translational modifications or inaccuracy of the prediction model, which does not consider the 3D structure.

A 3D model of Hum j 6 was generated using Swiss Model and showed an almost entirely alpha helical protein with an up-and-down 4-helical antiparallel bundle incorporating 5 cysteine residues. In the model, C7 and C14 as well as C112 and C72 are in close structural vicinity, while C134 locates in the C-terminus of the protein (**Fig. 3A**).

Only 3 PMEI allergens from plane tree pollens as well as kiwi have been described to date. The sequence of HJ PMEI, Hum j 6, identified in this study, shares only 33.3% of the amino acid sequence with both Pla or 1 (Oriental plane) and Pla a 1 (London plane). It also shows 28.8% sequence identity with Act d 6 from kiwi fruit (**Fig. 3B**).

IgE reactivity of Hum j 6

Recombinant proteins were produced using *E. coli* and *Pichia* expression systems (**Fig. 4A**). The *E. coli*–expressed Hum j 6 with an additional 26 amino acids at the N-terminus (MGHNHNHNHNHNHNAAGDDDDKASVD-) derived from the vector should have 171 amino acids with a molecular weight of 17.985 kDa, while *Pichia*-produced Hum j 6 that is His-tagged at the C-terminus (-HHHHHHAAAN) has 155 residues with a molecular weight of 16.263 kDa. Approximately 6-fold more recombinant protein (3.52 mg/L in bacterial culture) was obtained from inclusion bodies than the soluble fraction (0.59 mg/L) of *E. coli*. For *Pichia*

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INH_B08_T7      MDLISSTCSKALYKDLCEKTLRADSSSSGAKVDGLAKIALKAASSSAKSIQGQITTLLKT 60
INH_C16_T7      ....................................T..................... 60
INH_B16_T7      ........................................................... 60
INH_D20_T3      ........................................................... 60
INH_A06_T7      ........................................................... 60
INH_B20_T7      ...........................................................S.... 60
INH_D02_T3      ...........................................................I.... 60
INH_D01_T3      .....R...................................................... 60
INH_D08_T7      ........................................................... 60
INH_A18_T3      ........................................................... 60
INH_A01_T7      ............F.............................................. 60
                ***** ******:********************** ***************** ****

INH_B08_T7      GKDKAVVAALKDCSENYSGANEQLGDSLKAMVAKRYSDVNTWVTAAMTDGDSCEDGFKSG 120
INH_C16_T7      ........................................................... 120
INH_B16_T7      A.................V..................................... 120
INH_D20_T3      A.....................................M................. 120
INH_A06_T7      A.......................................................... 120
INH_B20_T7      ........................................................... 120
INH_D02_T3      ...........................................................V..... 120
INH_D01_T3      .R......................................................... 120
INH_D08_T7      .R......................................................... 120
INH_A18_T3      ..........R................................................ 120
INH_A01_T7      ........................................................... 120
                .:********:********.********************** ********* ******

INH_B08_T7      TSPLTKANTNFSQLCSNVLAITNQLS 146  [146/146, 100%] (32/54, 59.26%)
INH_C16_T7      ........................... 146  [145/146, 99.3%] (1/54,  1.85%)
INH_B17_T3      ........................... 146  [144/146, 98.6%] (1/54,  1.85%)
INH_D20_T3      ........................... 146  [144/146, 98.6%] (1/54,  1.85%)
INH_A06_T7      ........................... 146  [145/146, 99.3%](13/54, 24.07%)
INH_B20_T7      ........................... 146  [145/146, 99.3%] (1/54,  1.85%)
INH_D02_T3      ........................... 146  [144/146, 98.6%] (1/54,  1.85%)
INH_D01_T3      ........................... 146  [144/146, 98.6%] (1/54,  1.85%)
INH_D08_T7      ................S.......... 146  [144/146, 98.6%] (1/54,  1.85%)
INH_A18_T3      ........................... 146  [145/146, 99.3%] (1/54,  1.85%)
INH_A01_T7      ........................... 146  [145/146, 99.3%] (1/54,  1.85%)
                ****************.*********
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Fig. 2. Polymorphism analysis of Hum j 6. The percentage and frequency of each isoform are shown in parentheses.

expression, 1.704 mg/L recombinant protein was obtained from the culture media as it was secreted. The purity of recombinant proteins was at least 80% as shown by SDS-PAGE. Interestingly, *E. coli*-expressed Hum j 6 appeared at 17 kDa, but *Pichia*-expressed one appeared at 13 kDa in SDS-PAGE, suggesting the shift in molecular weight by phosphorylation.

Specific IgE to purified natural Hum j 6 was recognized by 86.4% (19/22) of *HJ* pollinosis patients, while recombinant Hum j 6 expressed in *E. coli* (45.5%, 10/22) and *P. pastoris* (50.0%, 11/22) were detected with less frequency than the natural protein (**Fig. 4B**). Furthermore, the ELISA absorbance of recombinant proteins was considerably lower compared to that of the natural protein, indicating weak IgE reactivity capacity of both recombinant proteins.

A mutant C134A, Ala residue at position 134 instead of Cys, was produced in order to prevent the possible inappropriate disulfide bridge formation. Recombinant C134A appeared about

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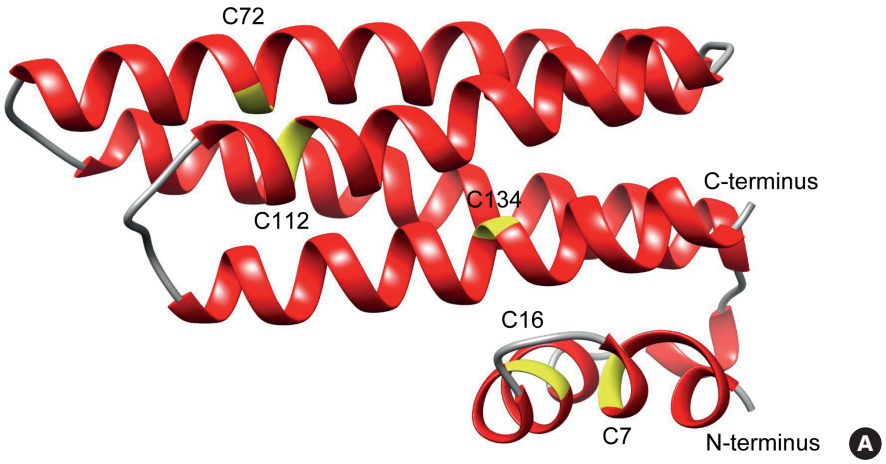


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Hum j 6     --DLISSTCSK------ALYKDLCEKTLRADSSSSGAKVDGLAKIALKAASSSAKSIQGQ 52
Act d 6     ENHLISEICPK------TRNPSLCLQALESDPRSASKDLKGLGQFSIDIAQASAKQTSKI 54
Pla a 1     -ADIVQGTCKKVAQRSPNVNYDFCVKSLGADPKSHTADLQGLGVISANLAIQHGSKIQTF 59
Pla or 1    -ADIVQGTCKKVAQRSPNVNYDFCVKSLGADPKSHSADLQGLGVISANLAIQQGSKIQTF 59
              .::.  * *          .:* ::* :*  *   .:.**. :: . *   ... .

Hum j 6     ITTLLKTGKDKAVVAALKDCSENYSGANEQLGDSLKAMVAKRYSDVNTWVTAAMTDGDSC 112
Act d 6     IASLTNQATDPKLKGRYETCSENYADAIDSLGQAKQFLTSGDYNSLNIYASAAFDGAGTC 114
Pla a 1     IGRILKSKVDPALKKYLNDCVGLYADAKSSVQEAIADFKSKDYASANVKMSAALDDSVTC 119
Pla or 1    IGRILKSKVDPALKKYLNDCVGLYADAKSSVQEAIADFKSKDYASANVKMSAALDDSVTC 119
            *  : :   *  :    : *   *:.* ..: ::   : :  * . *   :**: .. :*

Hum j 6     EDGFKSGT---SPLTKANTNFSQLCSNVLAITNQLS---  145, 100.0%
Act d 6     EDSFEGPPNIPTQLHQADLKLEDLCDIVLVISNLLPGSK  153,  28.8%
Pla a 1     EDGFKEKKGIVSPVTKENKDYVQLTAISLAITKLLGA--  156,  33.3%
Pla or 1    EDGFKEKKGIASPVTKENKDYVQLTAISLAITKLLGA--  156,  33.3%
            **.*:      : : : : .  :*    *.*:: *
```

B

Fig. 3. A 3D model (A) and sequence alignment (B) of Hum j 6. The 3D model of mature Hum j 6 was generated by Swiss Model (template 1XG2.1B). Alpha helical structures are depicted in red, and cysteine residues are shown in yellow. Amino acid sequence alignment of allergenic invertase/pectin methylesterase inhibitors was performed using Clustal O. Full sequence information was obtained by RNAseq analysis. Hum j 6 (*Humulus japonicus*, *Humulus scandens*, Japanese hop), Pla a 1 (*Platanus acerifolia*, London plane tree, CAD20556), Pla or 1 (*Platanus orientalis*, Oriental plane, ABY21305), Act d 6 (*Actinidia deliciosa*, green kiwi fruit, BAC54964). Red colors indicate phosphorylation sites.
3D, three-dimensional.

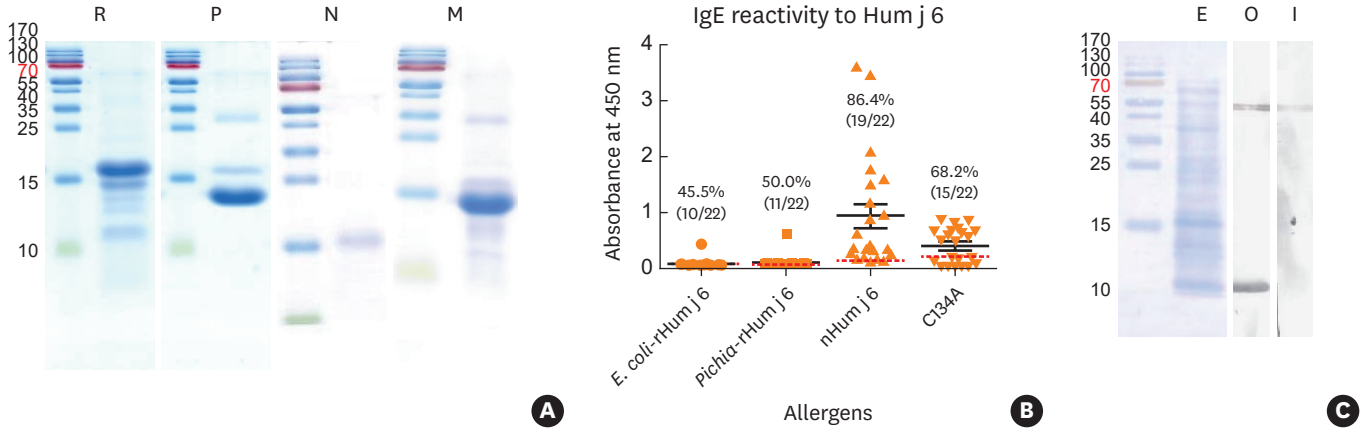

Fig. 4. IgE reactivity of Hum j 6. sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified proteins (A). IgE reactivity of purified proteins by enzyme-linked immunosorbent assay (B). Dotted lines indicate the cutoff value. Immunoblotting analysis of Hum j 6 (C).
IgE, immunoglobulin E; R, *Escherichia coli*-expressed recombinant Hum j 6; P, *Pichia*-expressed recombinant Hum j 6; N, purified natural Hum j 6; M, a mutant C134A; E, *Humulus japonicus* pollen extract; O, no inhibition; I, inhibition with natural Hum j 6.

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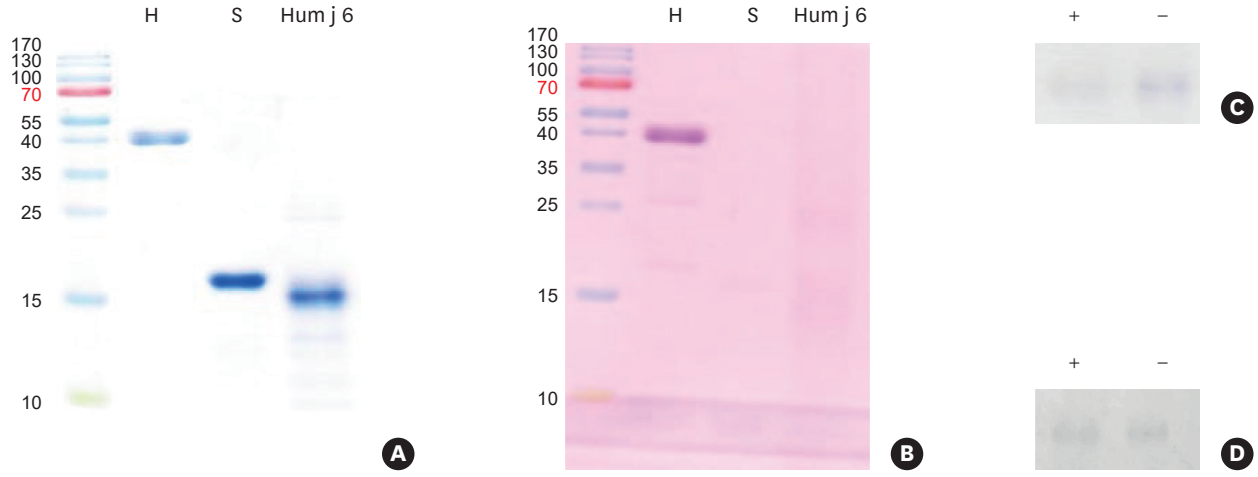

Fig. 5. Post-translation modification of Hum j 6. Purified Hum j 6 was separated by SDS-PAGE (A) and a glycoprotein stain was performed to detect any glycosylation (B). To determine the effect of phosphorylation on IgE reactivity, phosphatase-treated Hum j 6 and untreated Hum j 6 were run on SDS-PAGE (C) and IgE immunoblotting was performed (D).
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgE, immunoglobulin E; H, horseradish peroxidase; S, soybean trypsin inhibitor; I, Hum j 6; +, phosphatase treated Hum j 6; –, untreated Hum j 6.

15 kDa in SDS-PAGE gel, although its calculated mass was 17.812 kDa. The yield of C134 was 4.394 mg/L in bacteria culture. C134A showed improved IgE reactivity, being recognized by 68.2% (15/22), compared to the recombinant Hum j 6 with Cys at 134th residue (**Fig. 4B**). Still, IgE reactivity was lower than the natural Hum j 6.

Western blotting was performed to test the inhibition capacity of purified natural Hum j 6 in immobilized *HJ* pollen extract. Complete inhibition of IgE reactivity to the 10 kDa component was obtained by purified Hum j 6, corroborating the correct identification of PMEI as the major Japanese hop pollen allergen. In addition, partial inhibition of an IgE-reactive 50 kDa component was identified in gel analysis (**Fig. 4C**).

Post-translational modification of Hum j 6

No glycosylation of Hum j 6 was detected (**Fig. 5A and B**). Phosphorylation of at least 15 serine or threonine residues of Hum j 6 was detected by tandem MS analysis (residues in red in **Fig. 3**). Notably, phosphorylation did not affect IgE reactivity of Hum j 6, as no difference in IgE binding between native and phosphatase treated protein was observed (**Fig. 5C and D**).

DISCUSSION

PME is an important plant enzyme that is involved in the depolymerization of pectin, a major component of the plant cell wall. PMEI, homologous to the N-terminal propeptide of PME, functions in the regulation of PME.[12,13] PMEI is one of the highest represented proteins in the aqueous extract of fruits[14] and can be easily extracted at low ionic strength.[15] PME also plays a role in plant-pathogen interactions. Furthermore, PME can influence the structural quality of plant-derived food products.[16] PMEI can be used for the preparation of fruit juices and derivatives to stabilize foodstuff products by reducing methanol formation (PCR/IT2004/000390; WO2008104555).

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In this study, we identified the 10 kDa major allergen in SDS-PAGE from *HJ* pollen, which has been enigmatic, as a PMEI, Hum j 6. Only 3 PMEI molecules have been described as allergens originating from plane tree pollen and kiwi fruit. However, Hum j 6 shares low sequence identity (28%–33%) with these allergens, suggesting no or very limited IgE cross-reactivity.

We produced recombinant Hum j 6 using *E. coli* and *P. pastoris* expression systems. The recombinant proteins did not have comparable IgE reactivity with their natural counterpart, indicating the presence of structural differences between the proteins. There seems to be some difference in 3D structure of the proteins. Post-translational modification or 3D structure, possibly due to different disulfide bridging, could influence IgE reactivity. The cysteine at the 134th residue in Hum j 6, which is not conserved in Pla a 1 and Pla or 1 may interfere the formations of canonical disulfide bridges among conserved 4 cysteine residues. A mutant C134A showed improved IgE reactivity. However, it was not comparable to natural Hum j 6, indicating that there are some more structural differences.

Interestingly, glycosylation was detected for kiwi PMEI[14]: however our data showed that this is not the case for Hum j 6. The calculated pI value was different from the observed one in the 2D gel analysis. A considerable change in pI is often observed for proteins with a pI above 7.0 upon phosphorylation.[17] Phosphorylation may induce some structural changes of the molecule. In addition, the degree of phosphorylation can also influence the mobility on an SDS-PAGE gel in this study. No phosphorylation should occur in *E. coli*-expressed Hum j 6. Yeast-based phosphorylation is expected in *Pichia*-expressed Hum j 6, although this also showed weak IgE reactivity. More studies are needed to elucidate the detailed structural aspect of Hum j 6. Of note, the disulfide bridge pattern may determine the overall structure of Hum j 6, as was also shown for the crystal structure of kiwi fruit PMEI used as template for the 3D model.[18] Based on the close vicinity, we speculate that Hum j 6 has 2 disulfide bridges (C7–C14 and C112–C72); this, however, needs further structural investigation. In contrast, C134 might not be involved in any bridge formation, and interestingly, this cysteine residue is not conserved in both plane tree PMEI.

The molecular mass of Hum j 6 is a further interesting aspect. A 10 kDa component was detected as major allergen in a previous study,[3] and it was the target molecule for this investigation. Notably, a 15 kDa protein was purified, and it was able to inhibit the IgE-reactive 10 kDa band in immunoblot analysis. Phosphorylation of the protein, which provides a more negative charge to the protein, may influence the mobility in the SDS-PAGE gel. The calculated mass of the mature protein is 15.113 kDa. During the chromatographic procedure with alkaline buffers, the 15 kDa protein was detected. Sometimes, both 10 kDa and 15 kDa proteins having the same N-terminal sequences were detected after cation exchange chromatography of natural Hum j 6. The degree of phosphorylation thus seems to affect the mobility in the gel unless there is some degradation of the C-terminus.

Additionally, IgE reactivity to a 50 kDa protein was partially inhibited by natural Hum j 6. Hum j 6 (10–15 kDa) may bind to *HJ* PME (37 kDa). PMEI binds to the putative active site of PME, preventing substrate binding.[19] In our investigation, the observed IgE reactivity to Hum j 6 together with PME could have resulted in IgE reactivity to a 50 kDa component due to poor dissociation in the protein gel. In previous study, Japanese hop PME demonstrated only weak IgE reactivity[5]; however associated with PMEI the observed IgE binding as well as inhibition with purified natural Hum j 6 could be explained.

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Molecular biology enables allergists to characterize and produce recombinant allergens. Still, there are some limitations to overcome yet. CREATE and BSP090 projects had been initiated to establish and validate the major allergen quantifications using recombinant allergen and monoclonal antibodies for the better allergen standardization of allergen extracts. Now only 2 recombinant allergens Bet v 1 and Phl p 5 are adequate for these purposes and about to be implemented in the European Pharmacopoeia in 2023.[20] Various attempts to develop better immunotherapeutics superior to allergen extracts using recombinant molecules have not been successful.[21,22]

We have previously evaluated other allergen candidates and reported PR-1 protein, PME, polygalacturonase, and profilin as minor allergens of *HJ* pollen.[4,5] Recently, purified PR-1 was shown to be recognized by 76.6% of *HJ* allergic patients in China.[23] However, the intensity of IgE reactivity (observance at 450 nm was below 0.6) was not impressive and inhibition to the pollen extracts was 53.68% at the 10 μg/mL concentration. In this study, the IgE sensitization frequency to natural Hum j 6 was 86.4% among *HJ* pollinosis patients, and the inhibition assays by immunoblot suggesting that the Hum j 6 as the representative single and unique major allergen of *HJ*. So, this allergen could be applicable to component-resolved diagnostics with high clinical relevance and allergen-specific immunotherapy.

In conclusion, we identified the representative major allergen of *HJ* pollen, Hum j 6, a novel allergen belonging to the pectin methyl esterase inhibitor family, which was significantly modified after translation. There is little sequence similarity between Hum j 6 and identified allergens. Thus, these findings will therefore enhance the diagnostic panel and provide valuable insights into the understanding of Japanese hop pollinosis. Additionally, these results help standardize the allergen extracts and develop diagnostics and immunotherapeutics.

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