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Identification of probable pectinesterase as a major allergen of pollen of the Asian white birch (*Betula platyphylla*) in northern China

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Abstract

Background: Pollen of the Asian white birch (*Betula platyphylla*) is a major source of allergens in spring in northern China, yet little research on its pollen allergens has been done so far.

Objective: To analyze the *B. platyphylla* pollen allergen profile of patients from northern China and to identify the major pollen allergens in this patient cohort.

Methods: Sera from 35 Chinese patients with birch pollinosis were collected for this study. The IgE-binding proteins in *B. platyphylla* pollen extract were analyzed by IgE immunoblots. A novel major allergen was purified by cation exchange chromatography and affinity chromatography. Its IgE reactivity was evaluated by ELISA. The protein was excised from a 2D electrophoresis gel and subjected to ESI-QUAD-TOF.

Results: In our study cohort, the prevalence of IgE specific for Bet v 1 was 68.8% (n = 22/32) as measured by ImmunoCAP. In immunoblots, two major bands at around 17 kDa and 70 kDa were detected by IgE from 68.5% (24/35) and 65.7% (23/35) of sera, respectively. The 17 kDa band was identified as Bet v 1 by a monoclonal antibody to Bet v 1 and inhibition experiments with recombinant Bet v 1. The 70 kDa band was a polymorphic glycoprotein and 7 isoforms were found. The protein was identified as probably pectinesterase with a molecular weight of 66 kDa and a PI of 5.7.

Conclusion: A 66 kDa protein, probably belonging to pectinesterase family, was identified as a novel major allergen of *B. platyphylla* pollen in patients from northern China.

Key words: *Betula platyphylla*, birch pollen allergy, Chinese population, major allergen, pectinesterase

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Introduction

Birch pollen is the major allergen source in spring in the northern hemisphere, especially in Northern and Central Europe.[1,2] The prevalence of birch pollen sensitization is approximately 8% to 16% in the general populations in Europe.[2] To date, seven IgE reactive proteins in *Betula verrucosa* (European white birch) extract have been approved by the World Health Organization and

International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (www.allergen.org), named Bet v 1 to Bet v 4 and Bet v 6 to Bet v 8. Bet v 1 is a 17 kDa protein from the pathogenesis related protein class 10 (PR-10) family. Bet v 1 is recognized by 62-98% of birch allergic patients from different populations and defined as the major allergen in birch pollen.[3] In more than 90% of birch pollen related food allergies, Bet v 1 homologs are the primary associated allergens.[4] Bet v 2 to Bet v 4 and Bet v 6 to Bet v 8 are minor allergens in birch pollen and have less clinical impact.[3,5-8] Bet v 2 and Bet v 4 belong to the profilin and polcalcin families, respectively, and show broad cross-reactivity with many allergens from pollen, latex and plant food.[2,9,10]

Birch grows in many vegetation regions of China, mainly in the southwestern (Sichuan, Yunnan and Xizang), northern (Beijing, Shanxi and Neimenggu) and northeastern (Heilongjiang, Jilin and Liaoning) parts of China. In northern and northeastern China, *Betula platyphylla* is the common species of birch, as well as *Betula dahurica*, *Betula ermanii* and *Betula chinensis*. *Betula platyphylla* belongs to the order Fagales, family Betulaceae and genus Betula. According to the pollen concentration data from Beijing in 2014, the season of tree pollens from March to May, with an annual pollen index (API) of 9,614 grains/m^3/year,[11] consisting of several species, e.g. *Sabina*, *Betula*, *Fraxinus*, *Platanus acerifolia*, *Salix caprea*, and *Populus*. The API of *Betula platyphylla* was 2042 grains/m^3/year with a season ranging from March 15th to June 4th 2014, occupying the second position in API of spring tree pollen.[11] Thus, birch is an important elicitor to cause allergic rhinoconjunctivitis in spring. However, studies on birch pollen allergy are limited in the Chinese population and information on the prevalence of birch pollinosis in China based on large-scale population surveys is scarce. The prevalence of Bet v 1 has been reported to be 82.4% in 32 patients with positive reactions to birch pollen extract Bet v.[12] Our unpublished data show that the positive rate of Bet v 1 determined by ImmunoCAP is 60.7% (n = 221/364) in birch pollinosis patients mainly from northern China. We were interested to identify birch pollen allergens in patients with birch pollinosis especially those with a negative serology to Bet v 1. In the current study, we analyzed the allergen profile of birch pollen using sera from birch pollinosis patients in northern China and identified a 66 kDa IgE-reactive protein as a novel major allergen.

Methods

Study subjects

A total of 35 patients with birch pollinosis were recruited for this study from March 2014 to June 2015 at the Department of Allergy, Peking Union Medical College Hospital. The diagnostic criteria for birch pollinosis were (1) a convincing history of birch pollen allergy including allergic rhinoconjunctivitis (ARC) and/or asthma during the birch pollen season, generally from March to May; (2) positive intradermal skin test results to a spring tree pollen mixture containing birch, maple, oak, walnut and oilseed rape pollen (Allergen Manufacturing and Research Center, Peking Union Medical College Hospital, Beijing, China, wheal diameter ≥ 5 mm); (3) serum specific IgE to birch pollen (t3) ≥ 0.35 kU_A/L by the ImmunoCAP allergen system (Phadia 1000, ThermoFisher Scientific, Uppsala, Sweden). Exclusion criteria were ongoing or completed allergen-specific immunotherapy with birch pollen, and/or presence of perennial symptoms of ARC or asthma. Five subjects without any history of IgE-mediated reactions and tested negative to common aeroallergens (i.e. mite, mold, weed and tree pollen) served as healthy controls. All subjects signed informed consent forms prior to the serum sample collection from healthy controls (n = 5) and patients (n = 35). This study was approved by the ethics committee of Peking Union Medical College Hospital (No. PUMCH1103).

Serum specific IgE measurement

Serum specific IgE against birch pollen (t3) and its components, Bet v 1 (t215), Bet v 2 (t216), Bet v 4 (t220) and Bet v 6 (t225) were tested in a part of these patients by ImmunoCAP allergen system. The positive value was defined as detectable specific IgE ≥ 0.35kU_A/L.

Pollen extraction

Pollen of the Asian white birch (*Betula platyphylla*) was collected in April 2014 from a birch forest located in the Ar Horqin Banner, Nei Menggu (Inner Mongolia) Autonomous Region of China. Pollen extracts were prepared by two methods. (1) Five grams of pollen were suspended in 5 mL sodium phosphate buffer (6.7 mM HNa_2O_4P, 3.3 mM NaH_2PO_4, pH 7.4) and incubated in a water bath at 37°C for 30 minutes. The suspension was vortexed for 1 minute every 10 minutes, then centrifuged at 3000 × g for 10 minutes. The supernatant was filtered through 0.2 μm filters (Sarstedt, Germany). (2) Two grams of pollen were suspended in 20 mL 20 mM sodium acetate (pH 4.5) with a half tablet of protease inhibitor (Roche Diagnostics, Mannheim, Germany). The suspension was shaken overnight at 4°C, then centrifuged at 3000 × g for 10 minutes. The supernatant was filtered through 0.2 μm filters and concentrated to 5 mL by passage through a 10-K cutoff filtration membrane microconcentrator (Microsep, Millipore, Merck, Darmstadt, Germany) by centrifugation at 4000 × g at 4°C. The protein concentration was determined by the BCA Protein Assay Kit (ThermoFisher Scientific).

Electrophoresis and immunoblotting

Pollen extracts were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Following SDS-PAGE, proteins from pollen extracts were blotted to a nitrocellulose membrane (GE Healthcare, USA) and incubated with sera from birch pollinosis patients and healthy controls diluted 1:10. Bound IgE was detected by an HRP labeled goat-anti-human-IgE antibody (Kirkegaard & Perry Laboratories, Inc., USA) and the fluorescent signals were visualized by a gel image system.

Purification of an IgE-binding protein with the apparent molecular weight of 70 kDa

A protein with molecular weight around 70 kDa (B70) bound IgE from more than half of the patients' sera in immunoblots. To purify B70, cation exchange chromatography (SP Sepharose, GE Healthcare, USA) and HiTrap™ ConA 4B (GE Healthcare, USA) columns were used. Pollen extracts prepared by the second method described above were directly loaded onto the cation exchange chromatography column packed with 3 mL SP Sepharose resin (GE Healthcare, USA), washed with 3 column volumes of 20 mM sodium acetate (pH 4.5) and then eluted with a gradient by using 20 mM sodium acetate and 1M NaCl (pH 4.5). Fractions were collected and analyzed by SDS-PAGE and immunoblotting with patients' sera. Those containing the target protein were pooled, then assessed by immunoblotting, using a 1:500 diluted rabbit-anti-HRP antibody (Bethyl Laboratories, USA) as first antibody and 1:1000 diluted goat anti-rabbit IgG-AP (Invitrogen, USA) as secondary antibody. The secondary antibody was visualized with the BCIP/NBT AP Substrate Kit (Invitrogen, USA).[13] Binding of the anti-HRP antibody indicated that B70 could be a glycoprotein.[13] Thus, a HiTrap™ ConA 4B (GE Healthcare, USA) column was chosen for further purification. The target fractions were collected after analysis by SDS-PAGE, immunoblotting and by inhibiting polled patients' sera with purified B70 by preincubation for 1 hour.

Two-dimensional gel electrophoresis (2DE)

The purified B70 was dialyzed against deionized water for desalting overnight at 4°C and the protein concentration was determined by the BCA Assay. After acetone precipitation and centrifugation, the protein pellet which was precipitated from 0.5 mL of dialysate calculated to contain 150 ug protein was resuspended in IEF sample buffer (7M urea, 2M thiourea, 2% CHAPS, 0.5% IPG buffer pH3-7, 0.002% bromophenol blue, 65 mM DTT). The sample was then loaded onto a single IPG gel strip (pH 4-7) for isoelectric focusing, and then separated by SDS-PAGE in an 8% gel. The detailed protocol has been described previously.[14] The 2DE gels were subjected to immunoblotting with patients' sera.

ELISA

According to Gepp et al.,[15] microtiter plates were coated with 2 μg/mL purified B70 in 50 mmol/L sodium carbonate buffer overnight at 4°C, blocked with 3% BSA, incubated with individual sera from patients or healthy controls, and finally with AP labeled anti-human-IgE mouse antibody. Normalized OD values exceeding the mean value of healthy controls by more than 3 SDs were considered positive.

Mass spectrometry

Mass spectrometry was performed by BPI (Beijing Protein Innovation Co. Ltd, Beijing, China). In brief, spots of interest were excised from 2DE gels, digested by trypsin and analyzed by ESI-QUAD-TOF. The data were analyzed by the Mascot software (Matrix Science, London, UK) to identify the protein via obtained peptide sequences in Uniprot database and the Mass Spectrometry Protein Sequence Database (MSDB).

Immunoblotting-inhibition assay

Based on the results of Mass Spectrometry, B70 was identified as probable pectinesterase. Then, pectinesterase was recombinant expressed and immunoblotting-inhibition assay was performed between B70 and recombinant pectinesterase. The main methods of inhibition assay were the same to immunoblotting. The only different step was before used as the first antibody, the sera were pre-incubated with recombinant pectinesterase 100 mg/ml for 1 hours at room temperature.

Results

Demographic and clinical characteristics of the patients with birch pollinosis

Based on the inclusion criteria, 35 patients were enrolled (male = 19, female = 16) with a median age of 32 years (range 8-57 years). All patients presented with ARC during the birch pollen season, and 7 patients (20%) suffered from ARC combined with asthma. Among the 35 patients, 16 presented with weed pollinosis in autumn. Twenty-five patients (71.4%) experienced plant-food allergy, for 20 out of these patients manifested as oral allergy syndrome. The detailed clinical profiles are listed in **Table 1**.

IgE reactivity profiles to birch pollen and its allergens by ImmunoCAP

By using ImmunoCAP, all 35 patients were shown to have specific IgE to birch pollen (t3). IgE to individual allergens were assessed in part of the 35 patients. Bet v 1 (t215) was the most frequently recognized allergen with a prevalence of 68.8% (n = 22/32), followed by Bet v 2 (t216) with a prevalence of 36.8% (n = 7/19) (**Table 1**). No patients were positive for Bet v 4 (t220, n = 0/18) and Bet v 6 (t225, n = 0/16) (**Table 1**).

Serological evaluation of IgE to birch pollen extracts by immunoblot

In immunoblot analysis using sera from 35 patients, two major IgE-binding bands were detected (**Figure 1**). A 17 kDa band, consistent with the molecular weight of Bet v 1, was recognized by sera of 24 patients (68.6%) (**Table 1**). A band at molecular weight around 70 kDa (B70) was detected by sera from 23 patients (65.7%). Eleven sera reacted exclusively with the 17 kDa band, 10 sera exclusively with B70, and 13 sera with both of them. The sera from patient no. 32 showed no IgE-reactive band in the immunoblot. No bands were recognized by sera from healthy controls.

Table 1. Clinical and serological profiles of studied patients.

Patient No.	Gender	Age (years)	Birch pollinosis	Pollinosis in fall	Food allergy	Specific IgE KU_A/L					Immunoblot		ELISA (B70)	Specific IgE to other airborne allergen sources
						Birch	Bet v 1	Bet v 2	Bet v 4	Bet v 6	17 kDa (Bet v 1)	70 kDa (B70)		
1	F	40	ARC	ARC	OAS	49.2	29.9	0.07	0.29	0.06	+	-	-	mugwort
2	M	9	ARC	ARC	OAS	9.05	0.26	ND	ND	ND	-	+	+	-
3	M	28	ARC	-	OAS	29.3	21.4	ND	ND	ND	+	-	-	-
4	F	15	ARC, AA	ARC, AA	ANA	25.5	0.41	18.9	0	0.01	+	-	-	mugwort
5	F	27	ARC	-	OAS, ANA	100	37.3	25.4	0.03	0.05	+	+	+	mugwort
6	M	18	ARC	-	ANA	24.6	21.7	0.01	0.01	ND	+	+	+	mugwort
7	F	41	ARC	-	-	24.3	7.98	ND	ND	ND	+	+	+	mugwort
8	M	46	ARC	-	OAS	ND	27.6	0.01	ND	ND	+	-	-	mugwort
9	M	45	ARC	ARC	OAS	5.09	0.04	9.18	0.03	ND	+	-	-	mugwort
10	F	49	ARC, AA	ARC		20.8	14	0.01	0	0.01	+	+	+	-
11	F	50	ARC, AA	-	OAS	7.79	10.8	ND	ND	ND	+	-	-	-
12	M	16	ARC	-	-	18.9	0.04	ND	ND	ND	-	+	+	-
13	M	46	ARC	-	-	6.7	ND	ND	ND	ND	-	+	+	-
14	F	33	ARC	-	OAS	10.9	ND	ND	ND	ND	-	+	+	-
15	M	19	ARC	ARC	ANA	19.8	0.01	24.3	0	0	-	+	+	-
16	F	39	ARC	-	AA	21.6	18.3	0.01	0	0.02	+	-	-	-
17	M	33	ARC	-	OAS	4.89	5.78	0.01	0	0.01	+	-	-	-
18	F	32	ARC, AA	-	OAS	5.36	ND	ND	ND	ND	+	+	+	-
19	F	32	ARC	ARC	OAS	96.2	100	0.06	0.04	0.04	+	+	+	-
20	F	21	ARC	-	OAS, ANA	17.8	0.03	15.7	0.02	0.04	-	+	+	-
21	F	44	ARC	-	-	4.89	6.91	ND	ND	ND	+	-	-	-
22	M	31	ARC	ARC	OAS	1.37	32.2	ND	ND	ND	+	+	+	mugwort
23	F	57	ARC	-	OAS	82.1	84	ND	ND	ND	+	+	+	-
24	M	55	ARC	ARC, AA	-	5.57	6.35	ND	ND	ND	+	+	+	-
25	M	49	ARC	-	OAS	32	32.1	ND	ND	ND	+	+	+	-
26	F	34	ARC	-	-	100	88.6	ND	ND	ND	+	+	+	-
27	M	14	ARC	ARC	OAS, ANA	1.38	0.13	0.02	0.01	0.01	-	+	+	mugwort
28	F	48	ARC	-	OAS, ANA	69	70	0.02	0.02	0.03	+	-	+	-
29	M	8	ARC, AA	AA	ANA	6.21	0.14	4.16	0.06	0.11	-	+	+	mugwort
30	M	56	ARC	ARC, AA	-	6.9	8.06	0	0.01	0	+	-	-	mugwort
31	M	9	ARC, AA	ARC, AA	OAS	13.4	0.08	ND	ND	ND	-	+	+	mugwort
32	M	13	ARC, AA	ARC, AA	-	8.16	0.02	8.01	0.02	0.02	-	-	-	mite, mugwort
33	F	15	ARC	ARC	OAS	3.95	5.23	0.02	0	0.02	+	+	+	mold, mugwort
34	M	27	ARC	-	-	12	13.3	0.01	0	0	+	+	+	mugwort
35	M	30	ARC	ARC, AA	OAS	31.9	0.29	ND	ND	ND	-	+	+	-

M: male; F: female; ARC: allergic rhinoconjunctivitis; AA: allergic asthma; OAS: oral allergy syndrome; ANA: anaphylaxis; ND: not done.

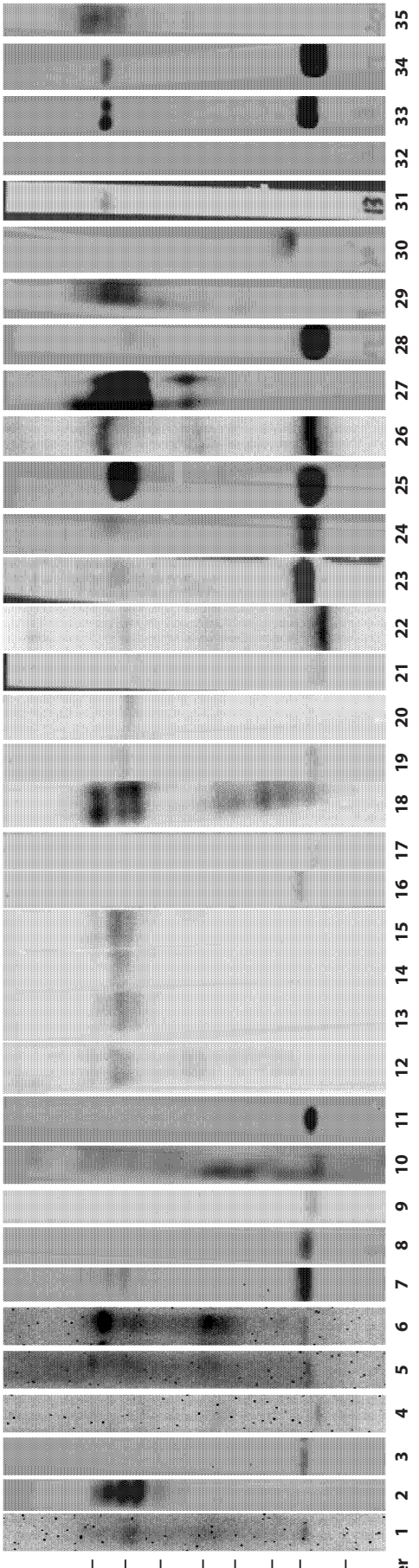

Figure 1. Immunoblot with 35 sera from birch pollinosis patients. A 17 kDa IgE reacting band was recognized by 24 sera and a 70 kDa protein band was bind with 23 sera.

To determine whether B70 was a Bet v 1 polymer, the monoclonal anti-Bet v 1 antibody BIP1 was used to inhibit patients' sera in immunoblotting. BIP1 recognized the 17 kDa band, but not B70 (**Figure 2a**). Immunoblot inhibition was performed by using recombinant (r) Bet v 1 as inhibitor. After preincubating sera from both 17 kDa band- and B70-positive patients with rBet v 1, IgE binding to the 17 kDa band was inhibited, and no effect on sera binding to B70 was observed (**Figure 2b**).

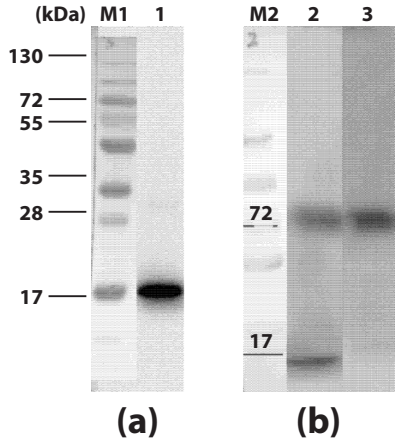

Figure 2. Cross-reactivity analysis between Bet v 1 and the 70 kDa protein. (a) In immunoblot, BIP (monoclonal antibody to Bet v 1) can bind with 17 kDa band but not 70 kDa band (lane 1). (b) A patient's sera recognized to both 17 kDa band and 70 kDa band by western blot (lane 2). After preincubated with recombined Bet v 1, the sera still bound to the 70 kDa band (lane 3).
M1: marker 1, figure 2a used marker 1. M2: marker 2, figure 2b used marker 2.

Purification of B70 from pollen of B. platyphylla

For a better yield of B70 from pollen, sodium acetate (pH 4.5) was selected as the method of extraction. The protein composition of birch pollen extracts prepared by the two methods was analyzed by SDS-PAGE. The concentration of B70 using the second method was much higher than that using the first method (**Figure 3a**). By immunoblotting, a polyclonal anti-HRP antibody was able to bind to B70, which indicated that B70 was a glycoprotein (**Figure 3b**). Thus, the purification of B70 was done in two steps, cation exchange chromatography and HiTrap™ ConA 4B column, (**Figure 3c**). The purified protein could to a great extent inhibit the binding between the 70 kDa protein band in birch pollen extract and IgE of a patients' serum pool (**Figure 3d**). The IgE-binding properties of the purified B70 were further detected by ELISA with sera from 35 patients and 5 healthy controls. Twenty-four (68.5%) showed positive reaction with purified B70 (**Figure 4**), which was almost consistent with the results of the immunoblot.

Identification of B70 as a pectinesterase

The polymorphism of B70 was investigated by 2DE and immunoblot with patients' sera pool (**Figure 3e and f**). Seven protein spots were separated in a range of isoelectric point (pI) values from 5 to 6, then excised from the gel and analyzed by ESI-QUAD-TOF. Two peptide sequences EGVYEETVR and KNVVFIGDGMGK were obtained from all seven spots. After peptide sequence alignment, these two peptide sequences showed 3% sequence coverage with a probable pectinesterase/pectinesterase inhibitor (UniProtKB: accession Q8L7Q7) (**Figure 5**). This protein has a molecular weight of 66 kDa and the calculated pI value of 5.71. N-terminal amino acid sequencing by Edman degradation of the purified protein failed, indicating blockage of the N-terminus.

Immunoblotting inhibition showed that recombinant pectinesterase completely inhibited the recognition of B70 by patients' sera (**Figure 3g**).

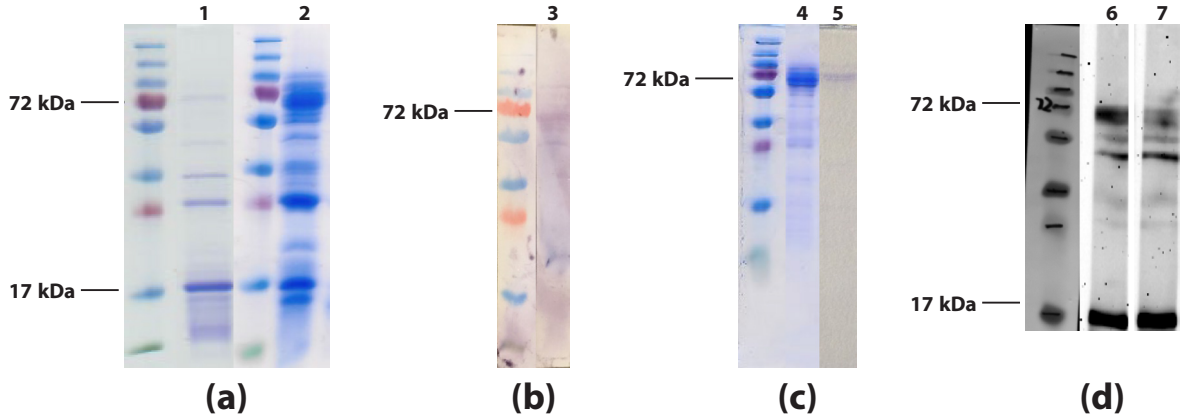

Figure 3. Purification of the 70 kDa protein (B70). (a) Birch pollen protein extracted by sodium phosphate buffer (lane 1) and sodium acetate buffer (lane 2). (b) Immunoblot of anti-HRP antibody with birch pollen extract (lane 3). (c) Purification of B70 by cation exchange (lane 4) and HiTrap ConA 4B (lane 5). (d) Immunoblot of patients' sera pool with birch pollen extract (lane 6) and inhibited immunoblot (lane 7), purified B70 serving as inhibitor. (e) Two-dimensional gel electrophoresis (2DE) of B70. (f) Immunoblot with patients' sera pool of B70 after 2DE. (g) After preincubated with recombinant pectinesterase, the patient's sera could not bind to B70 (lane 9).

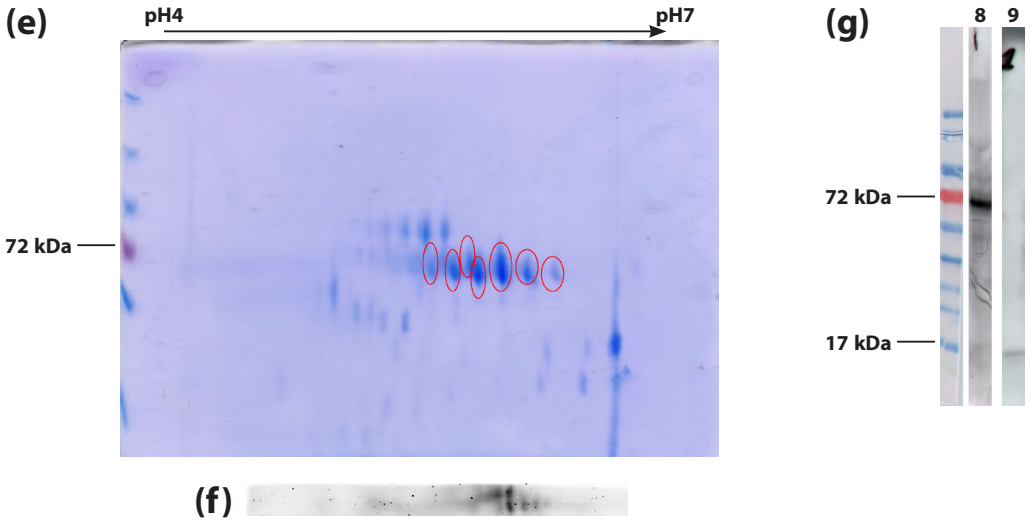

Figure 3. (Continued)

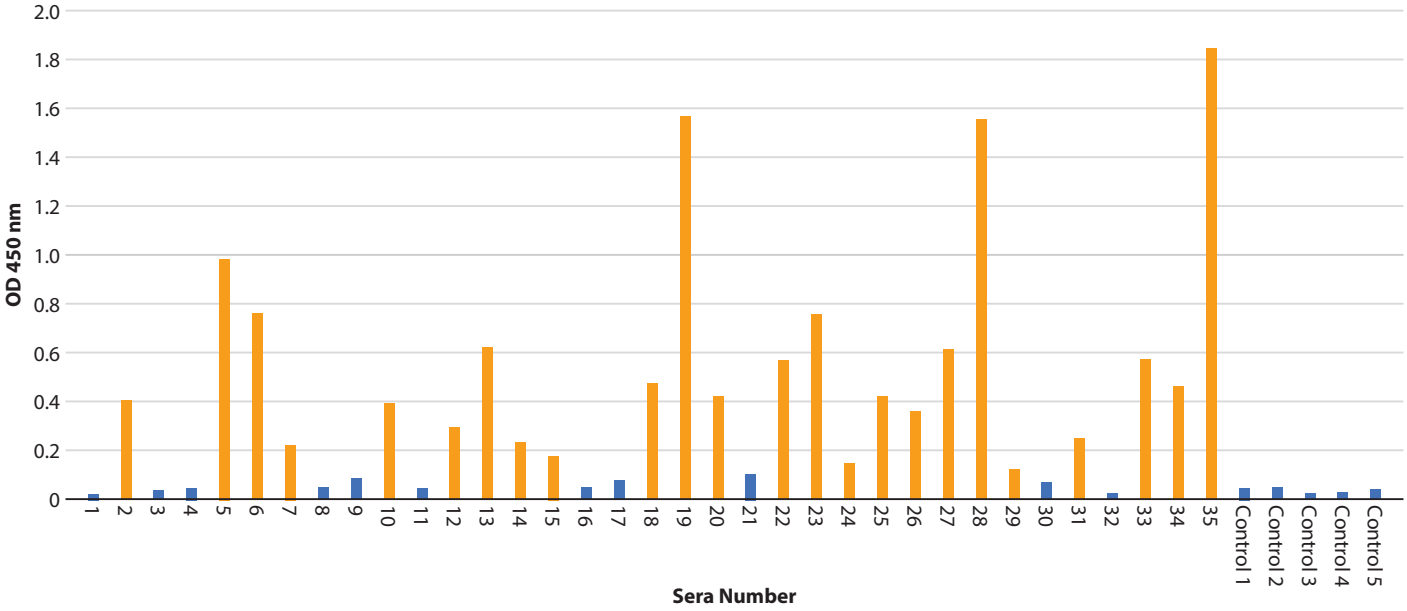

Figure 4. ELISA with 35 patients' sera and 5 controls of purified B70. Normalized OD values exceeding the mean value of healthy controls by more than 3 SDs were defined as positive.

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  1 MDSPTLPHSI SASSSTPFAS AAVKPHRNKL LSRNGILIII AASCILLLLI
 51 SLLIYATVSK SSRNHHNPSH QTPTSDDHPP PETPPSPPPI AQIRLACNAT
101 RFPDHCVASL SKPGQVPPDP KPVQIIHSAI SVSYENLKSG QSKIQSILDS
151 SAGNRNRTNI ATICLEILSY SQHRTESTDI AVTSGDIKDA RAWMSAALAY
201 QFDCWSGLKT VNDTKQVVDT ITFFEGLVNL TGNALSMMLS FDSFGDDVVS
251 WIRPATERDG FWEKAGPSLG SGTGTEASLG FPSGLTEDVT VCKNGGKDCK
301 YKTVQEAVDS APDTNRTVKF VIRIREGVYE ETVRVPFEKK NVVFIGDGMG
351 KTVITGSLNV GQPGMTTFES ATVGVLGDGF MARDLTIENT AGADAHQAVA
401 FRSDSDFSVL ENCEFLGNQD TLYAHSLRQF YKQCRIQGNV DFIFGNSAAV
451 FQDCDILIAS KHSKLEQGGA NNAITAHGRI DASQSTGFVF LNCSINGTEE
501 YMKEFQANPE GHKNFLGRPW KEFSRTVFVN CNLESLISPD GWMPWNGDFA
551 LKTLYYGEYK NTGPGSVRSS RVPWSSEIPE KHVDVYSVAN FIQADEWAST
601 TA
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Figure 5. Alignment of verified peptide sequences in B70 by mass spectrometry and probable pectinesterase/pectinesterase inhibitor 64 (UniProtKB: accession Q8L7Q7). Matched peptides shown in Bold Red.

Discussion

Based on the ImmunoCAP and immunoblot results, Bet v 1 was the most prevalent allergen for patients with birch pollinosis in our study group. The prevalence rate of Bet v 1 was calculated to be 68.7%, which is in accordance with our unpublished data obtained for a larger sample size (60.7%, n = 221/364), but lower than reported in another Chinese study (82.4%, n = 28/34) and most European studies.[2,12] The differences in birch species in different geographic areas and the exposure intensity to birch pollen may contribute to the observed differences in the prevalence of sensitization to Bet v 1.

We have identified a 66 kDa protein (B70) in *B. platyphylla* pollen as an allergen recognized by IgE from 65.7% sera from birch pollinosis patients in northern China. Therefore, B70 fulfills the requirement for being a major allergen and can be defined as a novel major allergen in the pollen of Asian white birch. By using ESI-QUAD-TOF, two peptide sequences in B70 were identified and could match with a probable pectinesterase/pectinesterase inhibitor 64 with coverage of 3%. The coverage between B70 and pectinesterase/pectinesterase inhibitor 64 was indeed poor. Thus, we aligned the two peptide sequences in standard databases online by BLAST to identify whether they were unique. Two known proteins were matched and they were pectinesterase/pectinesterase inhibitor 64 and putative pectinesterase/pectinesterase inhibitor 51. However, the molecular weight of putative pectinesterase/pectinesterase inhibitor 51 is 137.9 kDa, which is much higher than B70. The molecular weight of pectinesterase/pectinesterase inhibitor 64 is 65.478 kDa, which is similar to B70. Therefore, we identified B70 as probable pectinesterase/pectinesterase inhibitor 64. Pectinesterase/pectinesterase inhibitor 64 have two important regions. One is located in N-terminal section (residues 87-237), acting as autoinhibitory domain. Another is located in C-terminal section (residues 288-595), acting as catalytic domain. The verified peptide sequence in B70, EGVYEETVR and KNVVFIGDGMGK, are located in residues 326-334 and residues 340-351 respectively, which belong to pectinesterase. Furthermore, we expressed recombinant pectinesterase and performed immunoblotting-inhibition assay. The recombinant pectinesterase could inhibit the recognition between patients' sera and B70.

Mahler et al. first reported pectinesterase as an allergen in birch pollen by immunoscreening of birch pollen cDNA libraries and IgE-binding by the recombinant protein from birch sensitized patients.[16] However, the sequence of pectinesterase from *B. verrucosa* pollen has not been disclosed and the prevalence of IgE-binding of this protein in birch-sensitized individuals is still unknown. Here, we confirm the results of this previous study. Combining the finding from birch pollen cDNA libraries[16] and our mass spectrometry results, we deduced that B70 was a pectinesterase, an under described allergen in birch pollen. B70 is glycosylated which could be inferred by binding to anti-HRP antibody. The polymorphism of B70 was analyzed by 2DE and seven individual spots in B70 were observed on the gel. All of the spots were at the same molecular weight of 66 kDa with pIs ranging from 5 to 6 and were identified as pectinesterase respectively. This indicates that all seven spots are isoforms of B70.

Pectinesterases are a class of ubiquitous enzymes which catalyze the demethoxylation of pectin, a major component of the plant cell wall, and release methanol. Pectinesterase is alternatively named pectin methylesterase (PME). Pectinesterases have been identified in higher plants, bacteria and fungi.[17] Their catalytic activity has been known to be involved in many processes in plants, such as cell wall decoration, fruit ripening, senescence and pathogenesis.[17,18] Pectinesterases display high polymorphism. The isoforms are encoded by a large multigene family and are differentially expressed in the variable plant tissues and development stages.[17] Pectinesterase isoforms vary in molecular weight, pI, degree of glycosylation and catalytic property.[17] In our study, the features of B70 were consistent with several features of pectinesterases, such as being a glycoprotein, and having seven isoforms.

The allergenicity of PMEs has been reported in many plant species including olive tree pollen (Ole e 11),[19] Russian thistle pollen (Sal k 1),[20] *Urtica dioica* pollen,[21] ash pollen (Fra e 11), kiwi fruit (Act d 7) and *Solanum lycopersicum* (Sola l PME) (http://www.allergome.org/). The sequence identity among these pectinesterase with allergenicity were from 26% to 78%. Gly327, Tyr329, Glu331, Val333 and Gly346 of verified two peptides in B70 showed high degree of conservation with Ole e 11, Sal k 1, Fra e 11, Act d 7 and Sola 1 PME, and Phe344 of verified peptides was conserved in B70, Ole e 11, Fra e 11 and Act d 7. The clinical relevance of PME and the manifestations of allergic symptoms has not yet been well established. It was inferred that pectinesterase in birch pollen might be associated with cross-reactivity between birch pollen and plant-foods.[16] However, in the current study, there was no difference in the prevalence of food allergy between patients reacting with Bet v 1 and those reacting with B70 (Chi-square test, $p > 0.05$).

Conclusion

To summarize, we analyzed the allergen profile of Asian white birch pollen recognized by a northern Chinese patient cohort with birch pollinosis. A 66 kDa IgE-reactive band in birch pollen identified as probable pectinesterase was recognized by 65.7% of sera and defined as a novel major allergen in birch pollen in our cohort.

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Conflict of Interests

The authors report no conflict of interests.

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Authors' contributions

- Yingyang Xu has made substantial contributions to conception and design, perform the experiments, acquisition of data, analysis and interpretation of data, and drafting the article.
- Barbara Gepp and Nina Lengger have made contributions to perform the experiments.
- Jia Yin and Heimo Breiteneder have made substantial contributions to took part in drafting the article revising it critically for important intellectual content;
  agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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