

Analysis of Allergen Components and Identification of Bioactivity of HSP70 in Pollen of *Populus Deltoides*

Wei Guo

Anhui Normal University

Xiaodong Zhan

Wannan Medical College

Feng Jiang

Wannan Medical College

Yilong Xi (✉ ylxi1965@126.com)

Anhui Normal University

Research

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Abstract

Background: Allergies caused by pollen from *Populus deltoides* are common, but the allergic components are still unclear.

Methods: The total proteins in pollen of *P. deltoides* were analyzed by proteomics, and the potential allergens were identified via the WHO/IUIS database and the allergenOnline database retrieval. The target protein was screened by bioinformatics and expressed in *Escherichia coli*. The biological activity of the expressed product was verified by animal experiments.

Results: 3929 total proteins in pollen of *P. deltoides* were identified, and 49 potential allergens belonging to 10 protein families were recognized by database retrieval. B9N9W6 protein of Hsp 70 family was screened by bioinformatics analysis and expressed successfully. ELISA showed that B9N9W6 can stimulate the immune system to produce specific IgE and promote the generation of IL-4. Flow cytometry showed that B9N9W6 can significantly stimulate the proliferation of CD4⁺ T cells and promote the polarization of Th2 cells. The pathological sections of mice lung tissues indicated that alveolar destruction was more severe in the B9N9W6 group than that of extract group, and there were more inflammatory cells infiltration, mucus exudation and bleeding.

Conclusion: B9N9W6 is an important antigenic substance in the pollen of *P. deltoides*. Due to the conserved structure of Hsp 70 family, more attention should be paid to the possibility of sensitization when HSP 70 from any pathogenic species is administered.

Background

Allergic asthma is a chronic airway inflammation disease, which characterized by chest tightness, shortness of breath, and coughing after exposure to allergens [1]. The incidence of allergic asthma has been increasing in recent years [2]. At least 300 million people suffer from allergic asthma worldwide [3], which is highest among children [4, 5]. The main pathogenesis of allergic asthma is the production of specific IgE antibody, chronic airway inflammation and airway hyperresponsiveness during the immune system response to allergens in the environment, accompanied by the imbalance of Th1/Th2 cells and other comprehensive factors [6–8].

Pollen from plants is an important source of air-borne allergens, which seriously affects the quality of life for people who is susceptible to allergies [9]. During the period of flower opening, pollen grains are released into the air to form biological aerosols; thus, individuals are inevitably exposed to pollen. *Populus deltoides* is widely cultivated in China due to its urban greening, windbreak, and sand-fixing berm. In May of each year, mature pollen of *P. deltoides* is densely suspended in the air, which causes a mass of pollen to frequently contact people's eyes, nostrils, mouth and skin, leading to tears, sneezing, itching and other symptoms. Our recent studies demonstrated that mature pollen of *P. deltoides* extract contains antigenic substances with strong sensitization. However, allergic components in *P. deltoides* pollen remain largely unclear.

The identification and purification of pollen allergens is of great significance for pollen allergy disease, especially in diagnosis and allergen immunotherapy (AIT). In recent years, proteomic techniques have become powerful tools for comprehensive allergen analysis, such as Par h 1 in *Parthenium hysterophorus*, and Art an 7 in *Artemisia vulgaris* were identified by this method [10, 11]. In China, proteomics was used to analyze and compare the possible allergens in mutants of *P. deltooides* [12, 13]. Wang et al. have analyzed the possible allergen components in *Populus Tomentosa* by proteomics [14]. However, a systematic experimental basis is lacking for the identification of allergens in *P. deltooides* pollen.

HSP (Heat Shock Protein) 70 domain protein has a subtle relationship with allergic diseases. Previous studies have found that Hsp70 is an important mediator to mediate allergic reactions and is capable of binding IgE antibodies in allergic patients [15]. The levels of Hsp70 are significantly elevated in patients with allergic rhinitis [16]. Interestingly, Hsp 70 is widely present in plant pollen as a pan-allergen, which could be responsible for a part of the allergenic cross-reactivity between proteins from different pollens and plant food [17]. However, the biological function of Hsp 70 remains largely unknown.

To screen and verify *P. deltooides* pollen allergens, we analyzed the total protein of pollen through proteomics. Then, the sequences of identified protein were compared with the confirmed allergen via relevant allergen database to identify the allergen components in pollen. After that, Hsp 70 was expressed in prokaryotic expression system and explored its biological function by animal models. As far as we know, this is the first report of comprehensive allergenic proteins and Hsp 70 biological function in pollen of *P. deltooides*.

Materials And Methods

P. deltooides pollen sample

The pollen samples of *P. deltooides* used in this study were collected at the Yangtze River embankment (Wuhu, China) from Apr 20 to May 20, 2018, and stored in a refrigerator at -80°C.

Experimental animal

A total of 30 SPF female BALB/c mice (6-weeks) were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (License number: 20170005030182). The animals were kept under Specific Pathogen Free (SPF) laboratory conditions in the Hangzhou Hibio Technology Co. Ltd, with the room temperature at 22–26°C, light and darkness for 12 hours, respectively. Animals were free to eat and drink.

Protein extraction

Protein preparation for proteomics was as follows: The pollen samples were treated with trichloroacetic acid with a final concentration of 20% at 4°C for 2 h, centrifuged at 12000 g at 4°C for 3 min, and the supernatant was discarded. The protein precipitate was washed three times with pre-cooled acetone and reconstituted with 8 M urea. Then, the proteins were analyzed by 12% SDS-PAGE. Protein preparation for

animal experiments was as follows: Dried pollen was extracted with PBS (solid-liquid ratio of 1 g: 20 mL) at room temperature (RT) for 24 hours. The extracted materials were precipitated at RT for 8 hours. The crude extracts were filtered through 20 µm filter paper. Then, the extracts were sterilized through a 0.22 µm strainer (Millex GP) and stored at 4°C. Finally, the protein concentration was measured by the BCA kit (Sangon, C503071-0250).

Proteomics analysis of *P. deltooides* pollen

Proteomics analysis was performed with reference to relevant study methods [18]. The main process was as follows: total protein was digested with trypsin, and the tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 column. The tryptic peptides were analyzed via LC-MS/MS, and. We map and annotate the obtained MS/MS data using Maxquant software (v1.5.2.8). Subsequently, all the annotated peptide data were classified by R software for gene ontology classification and KEGG signaling pathway enrichment. The thresholds for the identification of total proteins and potential allergens in *P. deltooides* pollen were determined by relevant literature [19].

Screening of potential allergens in the Hsp70 protein family

The potential allergens of the Hsp70 protein family were screened by following steps. First, we performed T/B cell epitopes prediction for potential allergens of the Hsp70 protein family using the Immune Epitope Database and Analysis Resource (IEDB) (<http://www.iedb.org/>). Then, Antigenicity of the HSP70 were predicted through Vaxijen v2.0 (<http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) (Threshold 0.4). The formula, molecular weight (MW), stability and isoelectric point (PI) for B9N9W6 were predicted via ExPASy (<http://web.expasy.org/protparam/>), and its solubility was analyzed by ProtScale (<http://web.Expasy.org/protscale/>). Thirdly, we performed sequence alignment with HSP70s through the known allergens reported in the WHO/IUIS database and AllergenOnline database, respectively. Finally, we used Swiss-model for homology modeling to screen out Hsp70 with the highest surface complementarity with IgE antibody.

Prokaryotic expression and purification of Hsp70

Availability of adequate amounts of the pure allergens is essential to further understand their molecular structures, which is a pre-requisite for the development of more efficacious allergen immunotherapy. The amino acid sequences and coding genes of Hsp 70 were retrieved from Uniprot database. The gene of Hsp 70 was synthesized and cloned into prokaryotic expression vector pET-28a (+). The expression of His-tagged recombinant Hsp 70 in transformant *E. coli* BL21 was induced by IPTG (final concentration = 1 mmol/L), and the expression products were analyzed by SDS-PAGE. Then, the bacteria were collected and disrupted by sonication. Hsp 70 was purified by Ni-NTA protein purification kit (Sangon, C600320-0001), and protein concentration was measured by the protein concentration assay kit (Sangon, C503071-0250).

Preparation of asthmatic model

All BALB/c mice were randomly divided into 3 groups (10 mice in each group): PBS group, pollen extract group, and Hsp 70 group. Mice in extract group and Hsp 70 group were injected intraperitoneally with 10

µg pollen extract or Hsp 70 [dissolved in 4% (W/V) Al(OH)₃ in PBS, endotoxin-free] on 0, 7, and 14 days, respectively, to sensitize. From the 21st day, 0.5 µg/ml pollen extract or Hsp 70 suspension was sprayed for excitation for 30 min/time for one week. The PBS group was replaced with PBS containing 4% (W/V) Al(OH)₃. Mice in each group were injected intraperitoneally with 0.8 mg BrdU (dissolved in PBS) before the last challenge. After 24 hours, blood was taken from the eye socket, left at 4°C for 2 hours, and centrifuged at 4000 g for 5 minutes. The collected serum was stored at -80°C.

Enzyme-linked immunosorbent assay (ELISA)

The levels of total IgE antibody and IL-4 in serum were measured by ELISA kits from Songon, Inc. and Jianglaibio (Shanghai, China) (Sangon, D721094 \ Jianglaibio, JL20266) according to the manufacturer's protocol.

Flow cytometry

The spleens of mice in each experimental group were aseptically collected, and splenic cell suspension was prepared. The proliferation of CD4⁺ T cells and the ratio of Th1/Th2 in spleen cell suspension were detected according to the reported method [20]. In brief, the Th1/Th2 ratio in the splenic cell suspension was detected by the Th1/Th2 flow cytometry kit (Lianke Biotech, No. 70-KTH201) according to the kit's instructions. Cell suspension was incubated for one hour at RT in the dark after adding Anti-mouse CD4-FITC antibody (Invitrogen, No. 11-0042-85) and BrdU-PE antibody (Invitrogen, No. 12-5071-41), respectively, and then determined by flow cytometry.

Histological analysis

After the mice lung tissues were embedded in paraffin, sectioned (5µm thick), and hydrated with a series of alcohol, stained with hematoxylin and eosin (H&E), and then the changes in lung tissue structure were observed with a Nikon Ni-E microscope. Images were captured with a Nikon DS-Ri2 camera.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Data analysis was performed with SPSS16.0 statistical software package. One-way analysis of variance (ANOVA) was used for the comparison of multiple sample means, and the least significant difference (LSD) method and Tamhane's T2 method were used for the comparison between the two samples. The statistical test level $\alpha = 0.05$.

Results

Definition of *P. deltoides* pollen proteome

In the present study, we have used workflow (Fig. 1) to classify its allergen families and to characterize new *P. deltoides* pollen allergen candidates. Mass spectrometry data of the *P. deltoides* pollen proteome showed that the protein mass was mainly concentrated within 150kD, and the protein sequence coverage was mainly below 40% (Fig. 2A). The quantitative analysis of peptide length showed that peptides were

mainly distributed in the length of 7–27 amino acid residues, and the number of peptides with length 7–17AA was relatively concentrated (Fig. 2B).

Analysis by LCMS/MS showed that a total of 3,929 different proteins were identified. Gene ontology revealed that in terms of biological processes, these peptides were mainly involved in the metabolic process (32.49%), cellular process (27.26%) and single-organism process (19.21%) (Fig. 3A). From the perspective of cell components, it mainly involves cell (38.83%), organelle (23.74%), membrane (16.63%) and macromolecular complex (16.09%) (Fig. 3B). As for molecular functions, 43.35% of peptides were involved in catalytic activity, and 43.32% of peptides were related to binding (Fig. 3C). The subcellular localization results showed that 37.47% of the peptides were located in the chloroplast, 28.86% of the peptides were located in the cytoplasm, and 14.07% of the peptides were located in the nucleus (Fig. 3D).

Enrichment analysis of the KEGG signaling pathway and domains contained peptides

Enrichment analysis on the 3929 identified peptides showed that the top 5 signal pathways that were significantly enriched were ribosome, carbon metabolism, biosynthesis of amino acids, citrate cycle, and proteasome (Fig. 4A). The domain enrichment results showed that the top three domains were Aldolase-type TIM barrel, Heat shock protein 70kD C-terminal domain and Translation protein beta-barrel domain (Fig. 4B).

Allergenic protein families and candidate allergenic proteins

Sequence alignment between the WHO/IUIS database and the allergenOnline database identified 49 potential allergens that belonged to 10 protein families (Table 1).

Table 1
The information of predicted allergenic proteins in the pollen of *P. deltooides*

Allergenic protein family	Protein accession	Identity (%)	Protein description	Sum
pathogenesis-related proteins	A9PHF7	Hev B 6 (95.2%)	Class 4 pathogenesis-related family protein	1
Profilins	A9P8N4	Hev b 8 (94.7%)	Profilin	1
Peroxidases	B9GYJ9	Che a 3 (61.8%)	Peroxidase	1
Seed storage proteins	A0A2K1ZNE2	Gly m Bd (62.2%)	Vicilin-like seed storage protein	3
	B9H8M2	Pis v 2 (78.9%)	11S globulin seed storage protein	
	B9HNV4	Ana o 2 (75.3%)	Vicilin-like seed storage protein	

Note: Only homologous allergen with the highest identity has been displayed.

Identity, homologous allergen in the AllergenOnline database. Sum, total potential pollen allergens identified in the family.

Allergenic protein family	Protein accession	Identity (%)	Protein description	Sum
Proteases	A0A2K1XV49	Der p 15 (57.3%)	Protease Do-like 2 Subtilisin-like protease	16
	A0A2K1ZSB7	Pen c 1 (57.6%)	Cysteine protease	
	A0A2K2AML0	Ana c 2 (77.0%)	ATP-dependent Clp protease	
	A9PA38	Der p 15 (56.6%)	Aspartyl protease Protein	
	A9PBM7	CPA63 (69.4%)	Protein aspartic protease in guard cell	
	A9PCK2	CPA63 (68.5%)	Aspartic protease family protein	
	B9GJE2	CPA63 (68.5%)	Aspartyl protease family protein	
	B9GR19	CPA63 (68.5%)	Aspartyl protease family protein	
	B9GWZ3	Per a 2 (61.1%)	Subtilisin-like protease	
	B9H185	CPA63 (63.5%)	Kunitz-type protease	
	B9H290	CPA63 (63.5%)	Subtilisin-like protease	
	B9HR73	CPA63 (68.6%)	Aspartyl protease	
	B9I8Y7	Pen n 13 (51.8%)	26S protease regulatory subunit 7 family protein	
	B9IKK0	Pen n 13 (51.8%)	26S protease regulatory subunit 7 family protein	
	B9MUK8	Cup s 1 (57.5%)	Subtilisin-like protease	
	U5GXJ8	Pen n 13 (51.7%)		
		CPA63 (62.9%)		
		Pen n 13 (56.9%)		
	Asp f 1 (59.6%)			
	Pen n 18 (57.2%)			

Note: Only homologous allergen with the highest identity has been displayed.

Identity, homologous allergen in the AllergenOnline database. Sum, total potential pollen allergens identified in the family.

Allergenic protein family	Protein accession	Identity (%)	Protein description	Sum
Expansins	A0A2K1Z7R5	Pas n 1(68.1%)	Expansin-like A2	5
	A9PEQ7	Sor h 1(55.0%)	Expansin family protein	
	B9H521	Lol p l (57.9%)	Alpha-expansin 11 family protein	
	U5FK15	Cyn d 1(60.8%)	Expansin S1 family protein	
	U5GIP5	Dac g 1(65.2%)	Expansin-like B1	
Calcium-binding proteins	A9P988	Jun o 4 (69.6%)	Probable calcium-binding protein	2
	A9PCU6	pollen allergen group 2	Calcium-binding EF hand family protein	
Inhibitory proteins	A9P9T5	Ani s 4 (64.0%)	Cysteine proteinase inhibitor	6
	B9GNE2	Api m 6 (79.5%)	Cysteine proteinase inhibitor	
	B9GX99	Ani s 4 (58.8%)	Cysteine proteinase inhibitor	
	B9H290	Cry j 1(56.1%)	Kunitz-type protease inhibitor	
	B9HSW8	Pla o 1 (63.3%)	Cell wall /vacuolar inhibitor of fructosidase	
	B9I4L9	Ole e 3 (75.9%)	Invertase/pectin methylesterase inhibitor family protein	
Polygalacturonase	B9GQC0	Zea m 13 (55.4%)	Polygalacturonase-like family protein	2
	B9H9W1	Cry j 2 (71.4%)	Polygalacturonase	

Note: Only homologous allergen with the highest identity has been displayed.

Identity, homologous allergen in the AllergenOnline database. Sum, total potential pollen allergens identified in the family.

Allergenic protein family	Protein accession	Identity (%)	Protein description	Sum
Heat shock protein 70	B9GEL5	Alt a 3 (62.6%)	Heat shock protein 70	12
	B9GJ14	Der p 28 (77.4%)	Heat shock protein 70	
	A0A2K1XBF6	Cor a 1 (49.6%)	Activator of 90 kDa heat shock protein ATPase homolog	
	B9HMG2	Pen c 19 (90.1%)	Heat shock protein 70	
	B9HMG7	Pen c 19 (88.7%)	Heat shock protein 70 cognate	
	B9HMG8	Pen c 19 (91.6%)	Heat shock protein 70 cognate	
	B9HN74	Pen c 19 (91.6%)	Heat shock protein 70	
	B9HTJ7	Pen c 19 (91.6%)	Heat shock protein 70	
	B9HV59	Pen c 19 (78.1%)	Heat shock protein 70	
	B9N9W5	Pen c 19 (78.1%)	Heat shock protein 70 cognate	
	B9N9W6	Pen c 19 (90.6%)	Heat shock protein 70 cognate	
	B9NBF4	Pen c 19 (90.6%)	Heat shock protein 70 cognate	
		Pen c 19 (70.9%)	Heat shock protein 70 cognate	
		Der p 28 (88.2%)	Heat shock protein 70 cognate	
	Pen c 19 (90.0%)	Heat shock protein 70 cognate		
	Pen c 19 (91.8%)	Heat shock protein 70 cognate		

Note: Only homologous allergen with the highest identity has been displayed.

Identity, homologous allergen in the AllergenOnline database. Sum, total potential pollen allergens identified in the family.

Screening of potential allergens in the Hsp70 protein family

All the Hsp70s were checked, and B9N9W6 was selected as a potential allergen to elicit host immune response due to its physical and chemical characteristics as follows. IEDB epitope prediction showed that had 3 T-cell epitopes and 4 B-cell epitopes with high affinity (Table 2, 3). The formula of B9N9W6 was $C_{3146}H_{5062}N_{880}O_{1001}S_{21}$, MW was 71 900, PI was 5.34, and antigenicity was 0.5714 (Threshold > 0.4), which was considered as a stable hydrophilic protein. Query through AllergenOnline database showed that B9N9W6 had a conserved Hsp70 domain. Protein homology analysis revealed that the Hsp70 family protein sequences were extremely conserved (Fig. 5). Secondary structure of B9N9W6 was dominated by

α -helix and β ladder (Fig. 6), and it might be a hetero-dimer according to the crystal structure on the surface (Fig. 7).

Table 2
Prediction of T cell epitopes in B9N9W6

Epitope peptide	IC ₅₀ (SMM)	IC ₅₀ (NN)
QDLLLLDVTPLSVGI	25	21.2
LNVLRIINEPTAAAI	99	19.1
LRIINEPTAAAIAYG	176	34.5

Note: The predicted output is given in units of IC₅₀nM for Stabilized Matrix Method (SMM) and Neutral-Network (NN) scoring. The lower number indicates higher affinity. Peptides with IC₅₀ values < 50 nM are considered high affinity.

Table 3
Prediction of B cell epitopes in B9N9W6

Epitope peptide	Position
GRRFSDPSVQSDMKHWPF	78–95
KASGVKNKITYTNDKGRGLGKDDIERMVQEAERYKAEDEKVKKKVEAKNA	499–547
NTVRDDKVGKLDPADKQKIEKEIEETIDWLDRNQLAEVDEFEDK	557–601
QGAGGDVPMGGGAQMPGGAYSKASSGGSGAGPKI	618–651

Note: Bepipred Linear Epitope Prediction 2.0 was selected to predict the B cell Epitope, which analyzed the sequence characteristics of antigen and used the size of amino acid residues to predict the B cell epitopes.

Protein expression and purification of B9N9W6

SDS-PAGE of B9N9W6 showed specific band with a MW at 70KD, and purified product showed a clear band at same position (Fig. 8). His-tag detection revealed that the protein purity was more than 90%.

B9N9W6 induced antibody and cytokine responses

In order to explore the effects of B9N9W6 on serum antibodies and cytokines, the levels of IgE and cytokine IL-4 in serum were detected by ELISA kit. The results were showed in Fig. 9. The IgE level in the extract group (13.83 ± 5.59 ng/mL) was significantly higher than that in the PBS group (7.58 ± 2.40 ng/mL, $P < 0.05$), but significantly lower than that in B9N9W6 group (25.76 ± 11.90) ng/mL, $P < 0.01$). In terms of IL-4 level, the IL-4 level in the extract group (120.08 ± 36.58 pg/mL) was higher than that in the PBS group (74.69 ± 25.30 pg/mL, $P < 0.05$), while the IL-4 level in the B9N9W6 group (255.24 ± 81.88 pg/mL) was the highest among all groups ($P < 0.01$).

B9N9W6 induced CD4⁺ T cell proliferation and Th2 differentiation

In order to evaluate the ability of B9N9W6 to stimulate the proliferation of CD4⁺ T cells, we detected the number of proliferating CD4⁺ T cells and their subsets by flow cytometry. The results showed that compared with the PBS group (12.84 ± 0.53 %) and extract group (18.10 ± 0.58 %), B9N9W6 significantly promoted the proliferation of CD4⁺ T cells (27.86 ± 1.07 %, $P < 0.01$) (Fig. 10A, C1). Compared with the PBS group (2.39 ± 0.34), the ratio of Th1/Th2 in extract group (1.75 ± 0.24) and B9N9W6 group (1.69 ± 0.21) had significantly lower ($P < 0.01$) (Fig. 10B, C2).

B9N9W6 induced allergic inflammation in the lung tissue of mice models

Compared with the PBS group, the extract group showed reduced lung tissue alveoli due to destroyed anatomical structure, and increased mucus in the respiratory bronchus (Fig. 11B). A large number of inflammatory cells were exudated to the lung field. Alveolar septum thickens due to edema while mucus and inflammatory cells appeared in the terminal bronchioles and its periphery (Fig. 11E, H). Compared with the extract group, a mass of red blood cells exudate was found in the B9N9W6 group, and the destruction of alveoli was further aggravated (Fig. 11C); Bullae formed, the collapsed alveolar cavity and exudate of erythrocyte were detected; Mucus and inflammatory cells were abundant in the terminal bronchioles (Fig. 11F, I).

Discussion

In recent years, allergic diseases caused by pollens have attracted much attention, especially in the plant species releasing pollens, specific IgE reactivity and the influence of air pollutants on pollen transmission [21–23]. Nevertheless, identification of allergenic proteins and their bioactivity have remained elusive. In this study, we detected 3929 distinct proteins in pollen of *P. deltooides* by proteomics. We performed systematic bioinformatics analysis of all identified proteins, including protein annotation, functional classification and functional enrichment. Through functional annotation, we found that the total proteins of *P. deltooides* pollen and *P. tomentosa* pollen have great difference in function [14]. By GO categorization, we found that the total proteins of *P. deltooides* pollen were significantly different from those of its two mutants in biological process, molecular function, and cellular component [12]. KEGG enrichment analysis indicated that these identified proteins were not only involved in organelle composition and biogenesis, but also in biological processes such as metabolism and synthesis. There are great differences in the ingredients and functions of pollen proteins among different species of the same genus, which provides a material basis for antigen screening.

Plant-derived allergens mainly belong to disease-related protein 10 (DRP-10), thomas protein-like protein (TLP), Non-specific lipid transfer proteins (nsLTPs), expansion proteins, calcium binding proteins and profilin protein families [24, 25]. These proteins are called pan-allergen in specialized terms such as

profilin, because the same family of proteins has a common antigenic determinant, and they can cause a wide range of cross-reactions [26]. Proteins of the same family share a common domain and are relatively conservative in structure, which caused the common allergen proteins can be identified in a variety of plants [27–29]. In this study, through sequence alignment in the database, we identified 49 potential allergens belonging to 10 protein families. The protease family and the Hsp70 family are the most abundant. These results indicated that *P. deltoides* pollen not only contained abundant protein components, but also easily had fruit-vegetable-pollen cross-reactive allergy syndromes.

Hsp represent a family of molecular chaperones that response to refolding proteins, protein trafficking, and cell signaling processes [30–32]. Hsp 70 is an important member of the Hsp family involved in stress response, which often used as a potential biomarker, therapeutic target, or modulator of inflammation [33, 34]. Furthermore, Hsp 70 is also involved leaf remodeling, flowering and disease resistance in plant [35–37]. A correlation between biological function and allergenic capacity of proteins related to stress response has not been clearly demonstrated. Studies had shown that luminal binding protein of Hsp 70 family in hazel pollen is a cross-reactive allergen [17]. C-terminal region of Hsp 70 of *Echinococcus Granulosus* is antigenic molecule inducing both B and T cell responses [15]. Coincidentally, a large number of proteins and potential allergens containing the Hsp70 domain were identified in this study, such as B9N9W6. Epitope prediction suggested that B9N9W6 might have antigenic activity. Sequence alignment showed that B9N9W6 was highly consistent with the amino acid sequences of known allergens Cla h 4, Der f 28, etc. Homology modeling for B9N9W6 found that its 3-dimensional structure was also highly similar which consists of two main useful realms separated by a hinge region (Fig. 6A, and 6B); which accorded with the structural characteristics of the Hsp70 family [38]. This remarkable conservation of both surface residues and main chain conformations in the Hsp 70 family plays an important role in conservation of IgE-binding epitopes[39].

Identification and purification of pollen allergens is of great significance both for the study of cross-allergic reaction and AIT. The immunoreactivity of Hsp 70 had been demonstrated in previous studies [17, 39, 40]. In this study, the bioactivity of B9N9W6 was detected by animal model. First, we demonstrated that B9N9W6 can stimulate the immune system to produce high levels of IgE antibodies and promote the production of IL-4 via ELISA. Allergen specific IgE antibody is a major cause of type I allergic diseases, such as asthma [41], IL-4 is an important proinflammatory factor secreted by Th2 cells to mediate allergic airway inflammation [42]. Meanwhile, the significant increase of IL-4 concentration in the B9N9W6 group indicated the imbalance of Th1/Th2 cells and the increase of Th2 cells. Secondly, we detected the proliferation of B9N9W6 to stimulate CD4⁺ T cells and their subgroup Th1/Th2 cells by flow cytometry. It was found that B9N9W6 could significantly stimulate CD4⁺ T cell proliferation and promote Th2 cell polarization. These results suggested an immunogenicity of B9N9W6, which were consistent with ELISA. All above results suggest that B9N9W6 may induce allergic inflammation in the airway of the mice model. To verify our hypothesis, the presence of inflammation was observed through H&E staining sections of the mice lung tissues. More inflammatory cells infiltration and mucus exudation were observed in the lung tissue of B9N9W6 group; the alveolar rupture was the most serious, and even caused

pulmonary hemorrhage. Therefore, we consider that under the same concentration, the sensitization of B9N9W6 was stronger than that of extract. There are three possible reasons for this result: 1) B9N9W6 might have a dominant T/B cell epitope, which can strongly stimulate the immune response (Table 2, 3). 2) As a member of the Hsp 70 family, B9N9W6 could enhance the activity of antibody-presenting cells (APCs) in the process of antigen processing and presentation, which is essential for the initiation and modulation of the asthmatic immune response [43]. 3) Hsp 70 is a positive regulator of airway inflammation and goblet cell hyperplasia in allergic airway inflammation [44]; the pathological findings of this study supported this view. Therefore, our findings suggested that B9N9W6 potentially induces allergen induced Th2 inflammatory responses.

Conclusions

In summary, This study employed the proteomics method to screen out 49 allergens from 10 protein families of *P. deltoides* pollen. Furthermore, B9N9W6 protein of HSP70 family was confirmed to have strong allergen activity and can induce allergic asthma in animal models. Our conclusions not only enrich the theoretical study of Hsp70 family in pollen allergens, but also provide reference for the study of cross-allergic reaction and immunotherapy of allergic diseases. In the future, we will focus on several candidate allergens that occurred in KEGG enrichment analysis, especially in Hsp70 mediated signaling pathways.

Abbreviations

Hsp: Heat shock protein; AIT: Allergen immunotherapy; DRP-10: Disease-related protein 10; TLP: Thomas protein-like protein; nsLTPs: Non-specific lipid transfer proteins; SPF: Specific pathogen free; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes

Declarations

Statement of Ethics

This study was conducted in strict accordance with the proposals of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the mouse in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Hangzhou Hibiotech Co., Ltd. (IACUC protocol number: HBFM3.68-2015). The pollen of *P. deltoides* used in this study were conducted in strict accordance with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Disclosure Statement

The authors declare that they have no conflict of interest.

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Author Contributions

Wei Guo and Yilong Xi conceived the idea and wrote the manuscript. Xiaodong Zhan depicted figures and analyzed data. Feng Jiang contributed for revision. Yilong Xi contributed for overall editing and supervision. All authors approved its submission.

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Figures

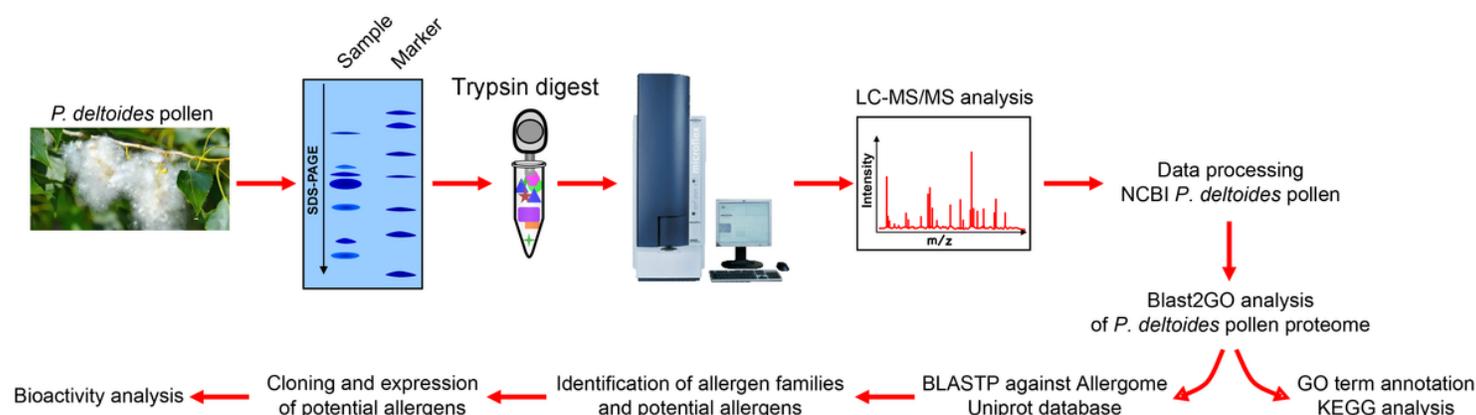


Figure 1

Workflow of the study to delineate the *P. deltooides* pollen proteome and analyze its bioactivity.

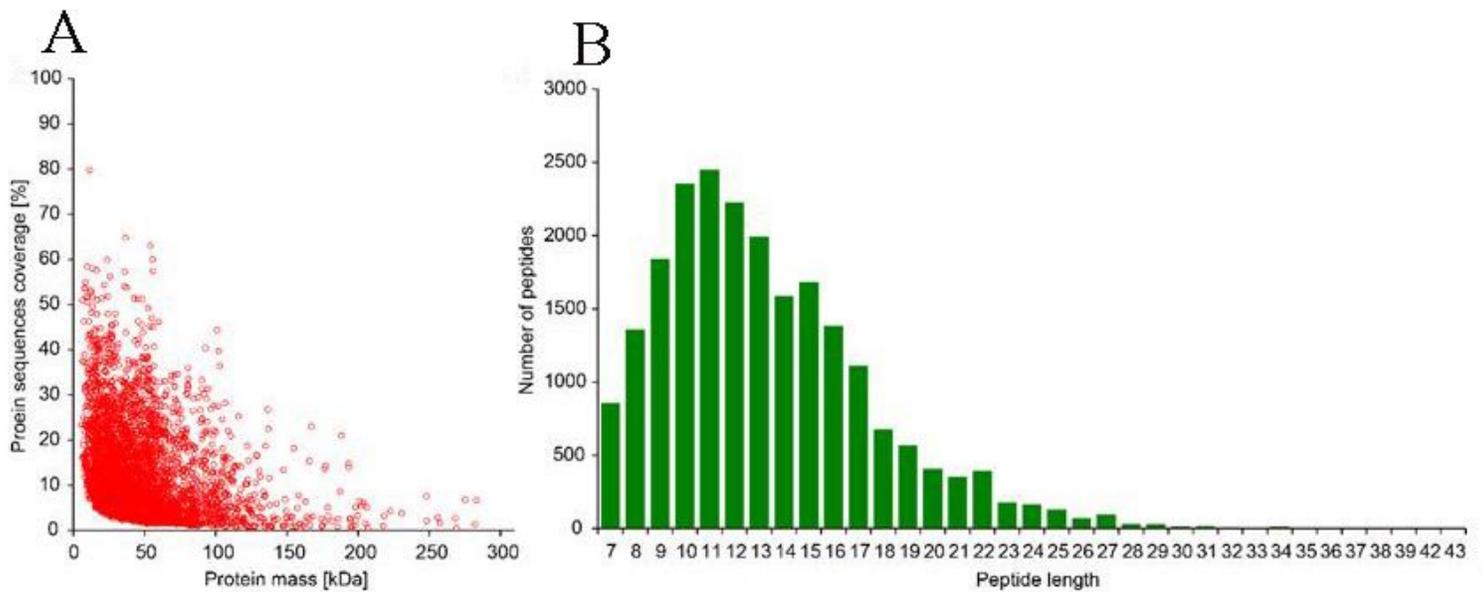


Figure 2

The characteristics of peptides in the proteome of *P. deltoides* pollen. A: Molecular weight and coverage. B: Length distribution.

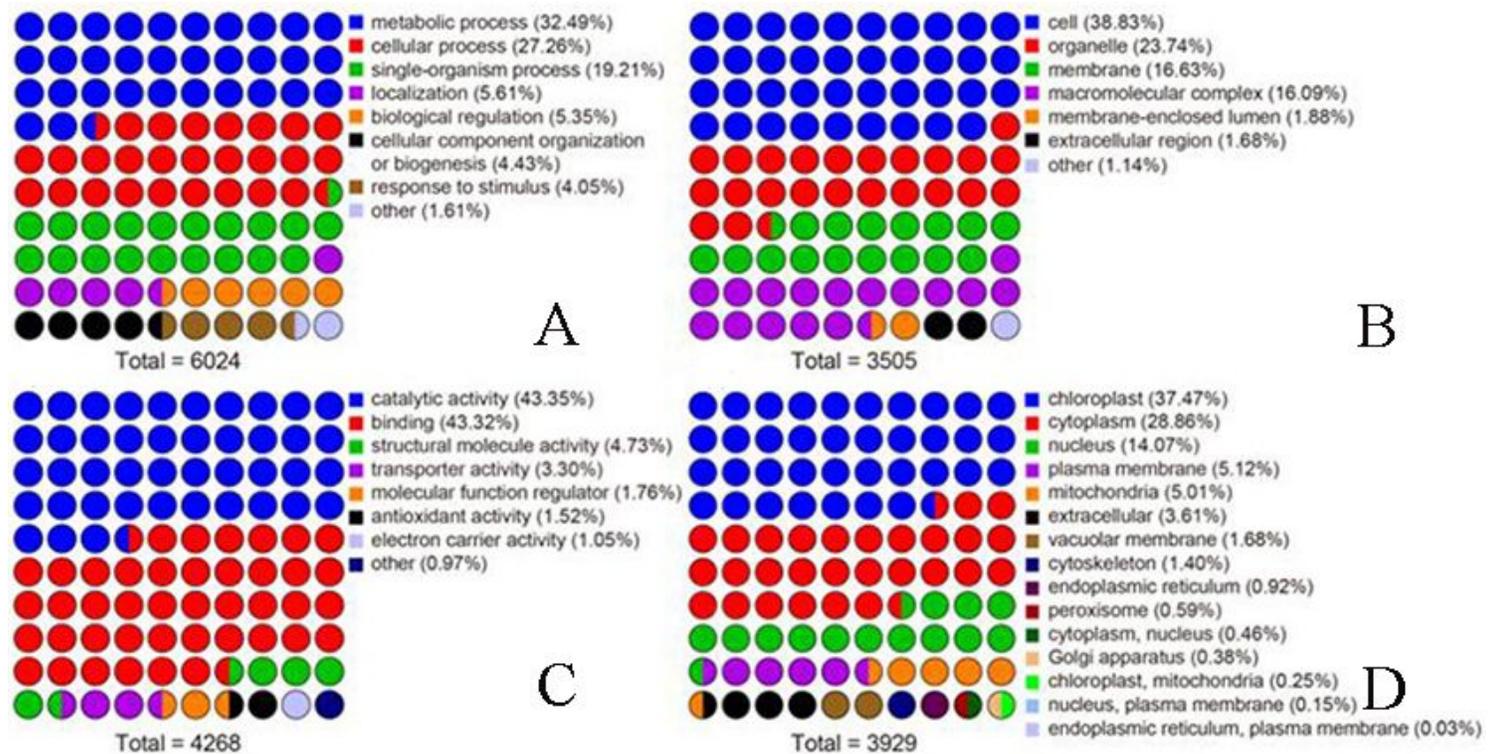


Figure 3

Gene ontology analysis of identified proteins. A: Biological process. B: Cellular components. C: Molecular function. D: Subcellular location.

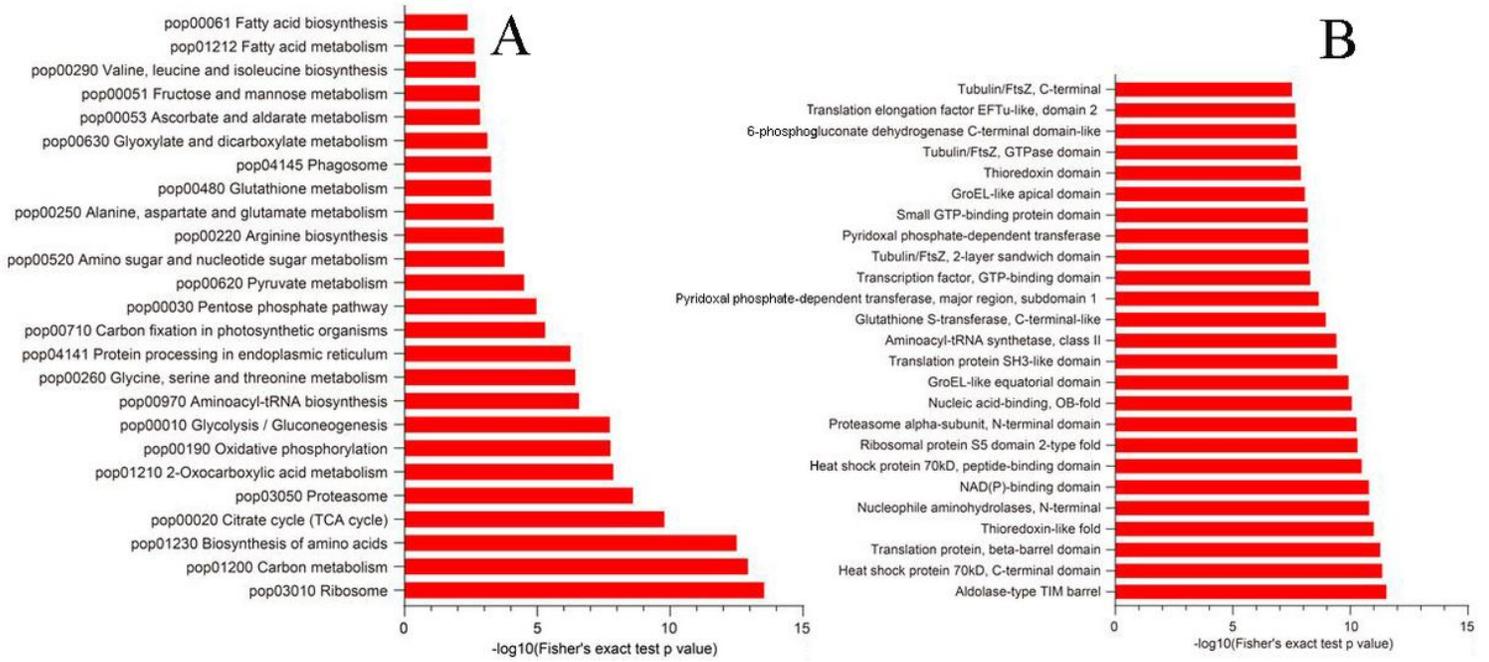


Figure 4

Enrichment analysis of the KEGG signaling pathway (A) and domains contained of peptides (B) in the *P. deltoides* pollen. The value of the horizontal axis is the negative log transformation of the significant P value ($P < 0.05$).

B9N9W6	MASKT-EGKA	IGIDLGTYS	CVGVWQNDRV	ELIANDQGNR	TTPSYVAFTD	TERLIGDAAK	NQVAMNPQNT	VFDAKRLIGR	RFSDPSPQSD	MKHWPFKVVP	DPADKPLIVV	QYKGEEKRFS	AEEISSMVLV	KMKE
Clah_4	-----MAP.IYRD.IF.....S.....S.....I.....H.....K.Q.AE.A.F.....IEK-.G..VTQ.	EF...T.D.T	P.....I..R.		
Der_f_28	P...LKAP.F.GS.N.S..T.	I.....DES..K.S.S	E-SG..KLE.	EF...R..W	P...A....				
Der_p_28	P...SKAP.F.GS.A.	I.....DE...K.S.S	E-SG...K.E.	EF...Q...W	P..V.A....					
Pen_c_19	..N.--KTP.F.HGK.T.D.A..A.S.N..N-DG..K.E.	EF...K.K.F	P.....						
Try_p_28	..N.--KTP.F.HGK.T.D.A..A.S.N..N-DG..K.E.	EF...K.K.F	P.....						
Alt_a_3	..N.--KTP.F.HGK.T.D.A..A.S.N..N-DG..K.E.	EF...K.K.F	P.....						
B9N9W6	IAEAYLGHAV	NNAVVTVPAY	FNDSQRQATK	DAGAIAGLNV	LRIINEPTAA	AIAYGLDKKA	SKSGEQNWLI	FDLGGGTFDV	SLLTIEEGIF	EVKATAGDTH	LGGEDFDNRL	VNHVFAEFR	KHKKDISANA	KALR
Clah_4	T...S...GT.I.....L.....Q E--K.....F.....S.....V.....DN.....S.....Q.K.	FG...MS.K	R...				
Der_f_28	T.....QK.	TDV.I.....V.....G G--K.....V.....DN.....S.....Q.K.	FG...MS.K	R...					
Der_p_28	T.....QK.	TDV.I.....V.....G G--K.....V.....DN.....S.....Q.K.	FG...MS.K	R...					
Pen_c_19	---...GT.I.....L.....T E--R.....S.....N.K.LTT..R..							
Try_p_28	T.....TT.	TS..I.....V.....T Q--K.....I.....D.....S.....Q.K.P.D.K	R...					
Alt_a_3	T.....TT.	TS..I.....V.....T Q--K.....I.....D.....S.....Q.K.P.D.K	R...					
B9N9W6	RLRTACERAK	RTLSSTTQTT	IEIDSLYEGI	DFYSTITRAR	FEEMNMDLFR	RCMEPVKCL	RDAAKIDKSHV	HEIVLVGGST	RIPKVQQLLQ	DFPNGKELCK	SINPDEAVAY	GAAVQAAILS	GEGNEK-VQD	LLLL
Clah_4SA..SF.....FTSN.....VQG.....	GN..G.RT.D.....S.....G.....K.VSP.....DTSS	STKE	I...		
Der_f_28S.....S.....H.....LCS.....	STL.....AL..KI	D.....I.K.SN.....T	DNSNN--K.				
Der_p_28S.....H..TLCS.....	STL.....AL..KI	D.....I.K.SN.....T	DNSNN--K.					
Pen_c_19AA..SF.....TS.....LCQ.....	GT...RV.S.....I.K.VS-DAN.DTSS	STNE	I...			
Try_p_28S..ASS.....LCA.....	STLD...S.L..NQII.E.N.....DESSE	--Q	I...				
Alt_a_3S..ASS.....LCA.....	STLD...S.L..NQII.E.N.....DESSE	--Q	I...				
B9N9W6	DVTPLSVIGE	TAGGVMTVLI	PRNTIPIPK	EQGVSTYSN	QTSVLVIQVE	GERAQTNDNN	LLGTFELKGI	PPAPRGPVQI	NVCDFIDANG	ILNWSAEDKA	SGVKNKITIT	NDKGRGLKDD	IERNVQEAER	YKAE
Clah_4	..A...L...A..K.....SET...F.....PG.....R..I..M.K..S.R.....E.T.L...M...LE.G	T.KT..V...S.EELAD..K..E.			
Der_f_28	..A...L...T..K.....T.T.T.A.PA.T.....M.....R...D.T.E.T.V...V.S	T.RQ.....S.A.K..N..QRE			
Der_p_28	..A...L...T..K.....T.T.T.A.PA.T.....M.....R...D.T.E.T.V...V.S	T.RQ.....S.A.K..N..QRE			
Pen_c_19	..A...L...P..K.....SET...F.....PG.....R.....K..T.E.T.L...M...SE.G	T.KS.....S.EELA..K				
Try_p_28	..A...L...A..K..S...QT.T.....PG.....M.....K..S.E.T.....V.S	T.KQ.....S.EK..S..RD.				
Alt_a_3	..A...L...A..K..S...QT.T.....PG.....M.....K..S.E.T.....V.S	T.KQ.....S.EELA..K				
B9N9W6	DEKVKKKVEA	KNALENYAYN	MRNTVRDDKV	GGKLDPADKQ	KIEKIEIETI	DWLDNRQLAE	VDEFEDKLE	LEGLCNPIIS	KMYQAGAGD	---VPMGG	---GAQMPGG	AYSKASSGGS	GAGPKIEEVD	
Clah_4	..AEAGRIQ.	..G..S..S	LK..S.P.	EE..SAE..E	TLTGA.DK.V	A.I.E..T.T	KE.Y.AEQ.Q	..SVA..VMM	..I.GAE..AP	---GG...Q	GAGAPPP	AG	DD..TV....	
Der_f_28	..RQRERIA.	..Q..A..FQ	LKS.MEEEA	KS..SEE.RK	TVLNKVD..L	R..S...D	KE..Y.HRQ.	..I.NA.R..MM	..I..QQQQQ	---HH.GAN	---SCGSNA	YPGYNGFKSN	ND..VV...N	
Der_p_28	..RQRERIA.	..Q..A..FQ	LKS.MEEEA	KS..SEE.RK	TVLNKVD..L	R..S...D	KE..Y.HRQ.	..I.NA.R..MM	..I..QQQQQ	---GA.GAN	---SCGSNA	YPGYNGFKSN	ND..VV...N	
Pen_c_19	..AEASRIQ.	..G..S..S	LK..ITEG	KLQMSDD..K	..DK.S.I.	S..N..T.	K..Y.SQQ.	..ATA..MQ	AA.GA..AP	---PQQRAD	ET	EKKDE..L		
Try_p_28	..QRDRIT.	R.T.S..FQ	IKSSIEEBAI	KS.ISEE.RK	..LDKV..M	K..S.A..K	..Y.HHR.	..AV...MT	..L.Q..AAP	GGGM.G.FPG	GFP	TGA.D	PADD--KAK	..S..T....
Alt_a_3	..AEARIS.S..S	L..LS.S.	DE..AG...	..LTA..DK.V	Q...D..T.T	K..Y.SQQ.	..VA...MM	..F.GAG.EGG	---M.G---	---MPGG.M	PGGAPGGAAG	DD..TV....	

Figure 5

Aligning of B9N9W6 with reported allergen amino acid sequences. B9N9W6 showed a high degree of homology with the Hsp70 family of allergens. "." represented completely identical amino acids. "-"

represented no amino acids.

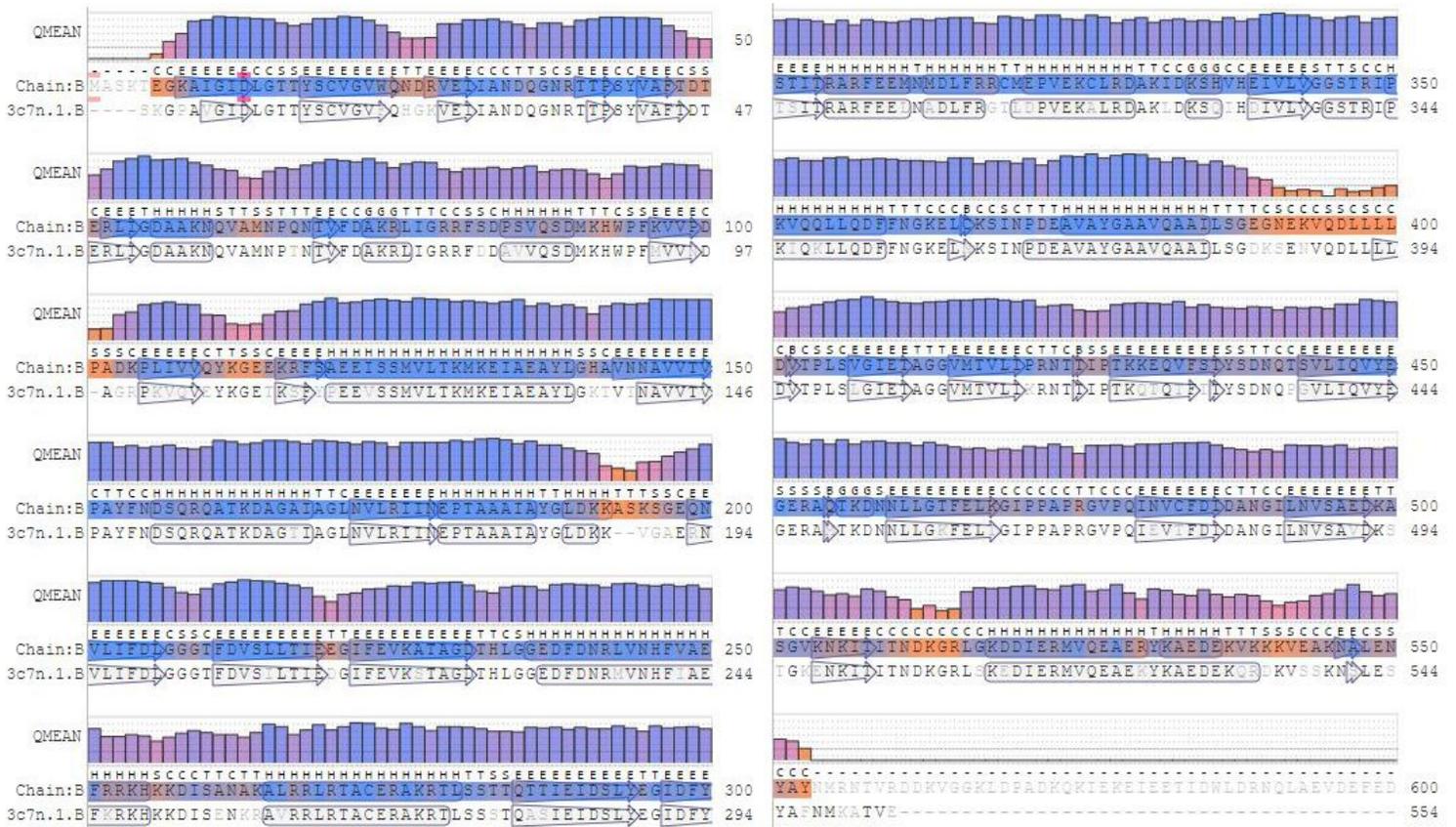


Figure 6

Aligning of B9N9W6 with template 3c7n.1.B amino acid sequences and secondary structure. The residues found in all protein polypeptide chains of the model are displayed in an interactive sequence display. Each residue is displayed by its one letter code below a bar chart displaying the QMEAN local quality estimation value. The secondary structure of the protein is displayed above each residue code as a single letter. B: residue in isolated β -bridge; C: loop or irregular; E: extended strand, participates in β ladder; G: 3-helix; H: α -helix; T: hydrogen bonded turn; S: bend.

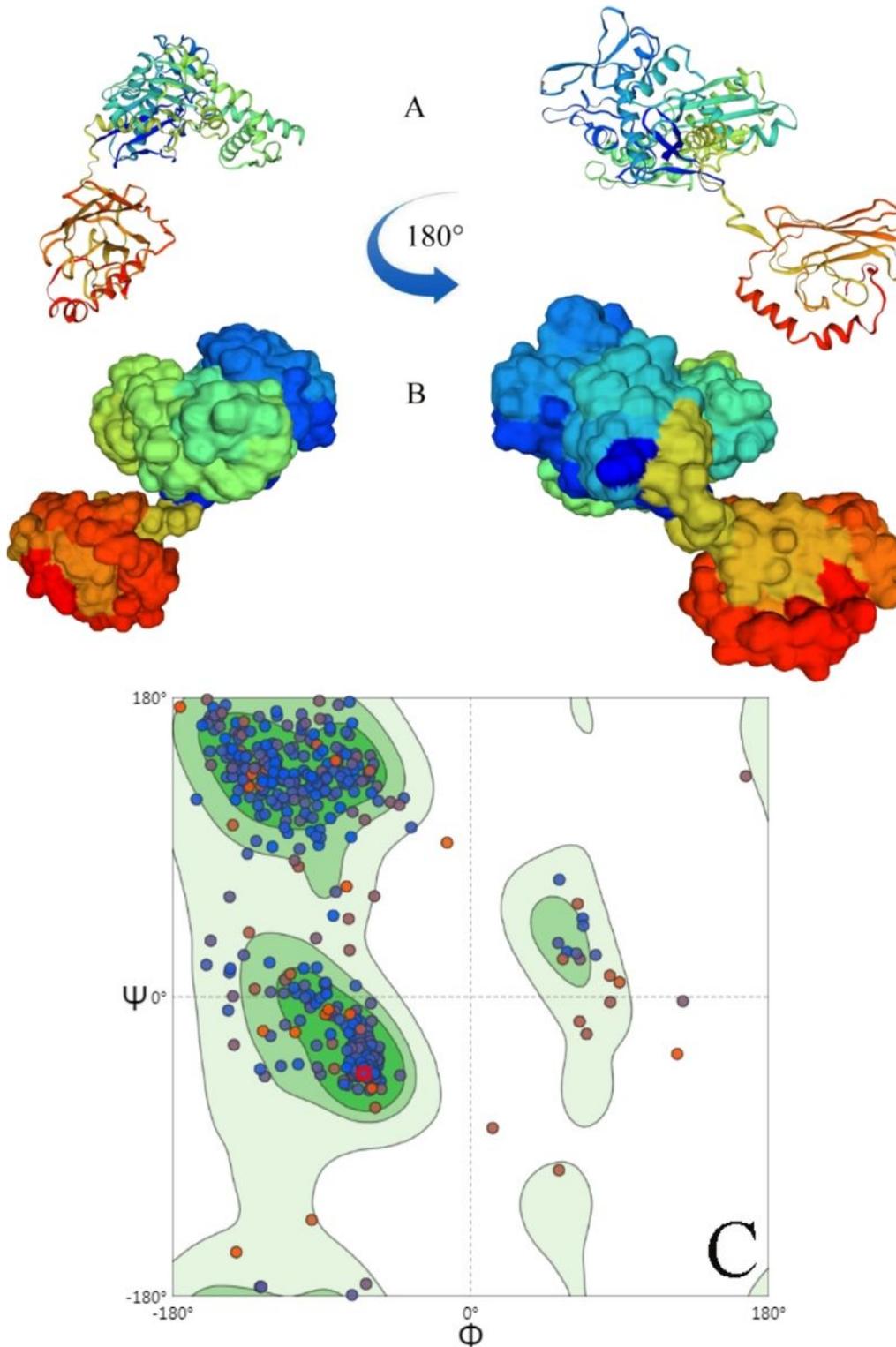


Figure 7

Molecular model diagram of B9N9W6 constructed by SWISS-Model. Template ID: 3c7n.1.B. QMEAN Value -0.86, GMQE Value 0.66 (The Qmean Value range is -4-0, and the closer it is to 0, the better the matching degree between the target protein and the template. The confidence range of GMQE Value is 0-1, the higher the value, the better the quality of the model). A: Ribbon diagram of B9N9W6. B: Surface structure diagram of B9N9W6. C: Ramachandran Plots of B9N9W6. The number of residues in favored

(90.11%), in allowed regions (7.61%) and in outlier regions (2.20%), which demonstrates the high quality of the model construction.

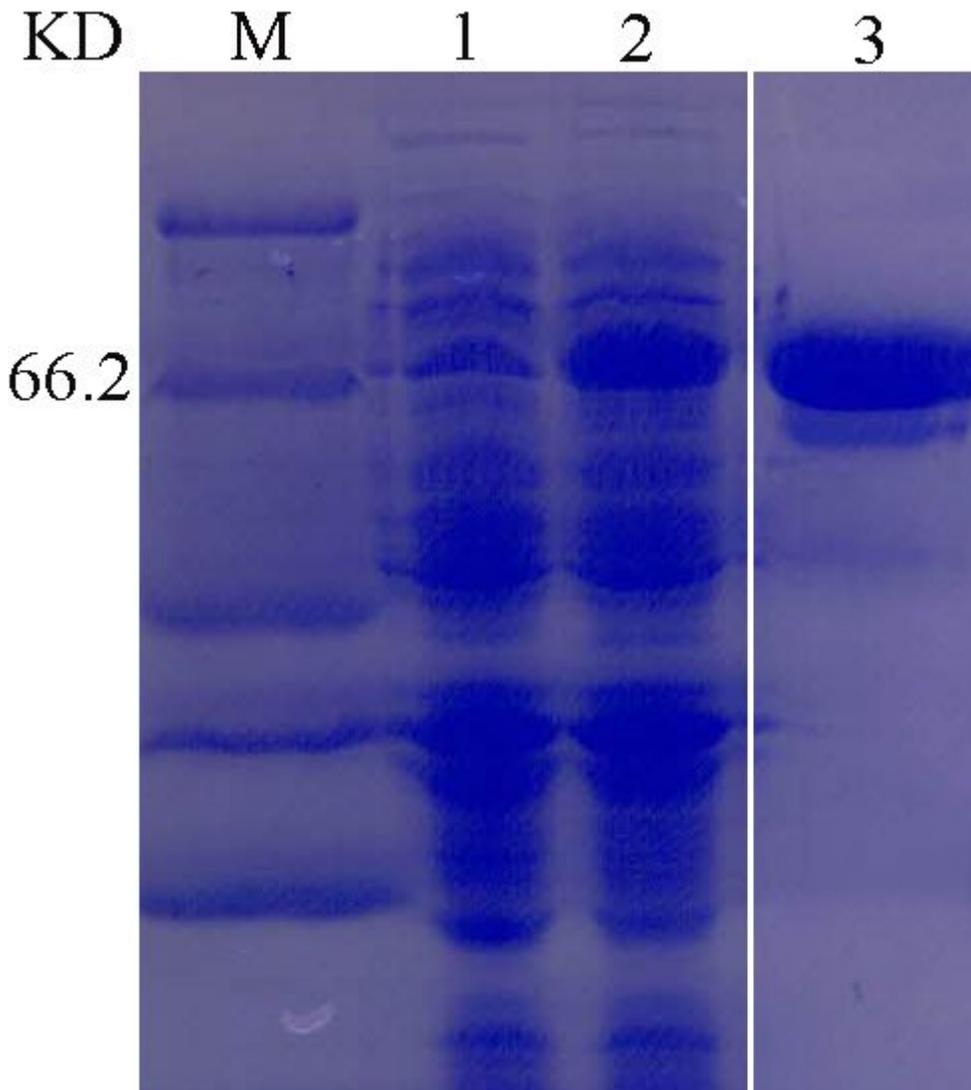


Figure 8

Prokaryotic expression and purification of B9N9W6. M: Protein Marker (C600525, Sangon Shanghai). 1: *E. coli* BL21- pET-28a (+). 2: *E. coli* BL21- pET-28a- B9N9W6. 3: purified product of B9N9W6.

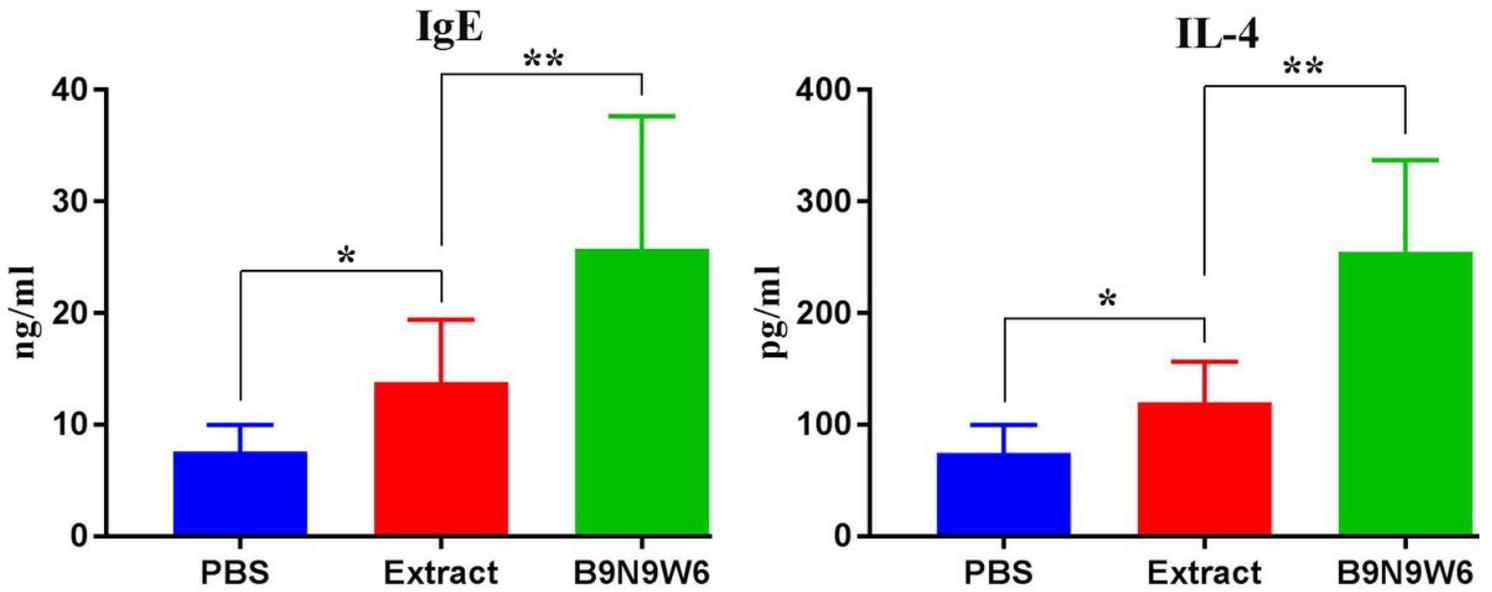


Figure 9

Effects of B9N9W6 on the expression of antibodies and cytokines. The vertical axis represents as the concentration of antibody or cytokine. Values are presented as the mean \pm standard deviation (SD). * P < 0.05; ** P < 0.01.

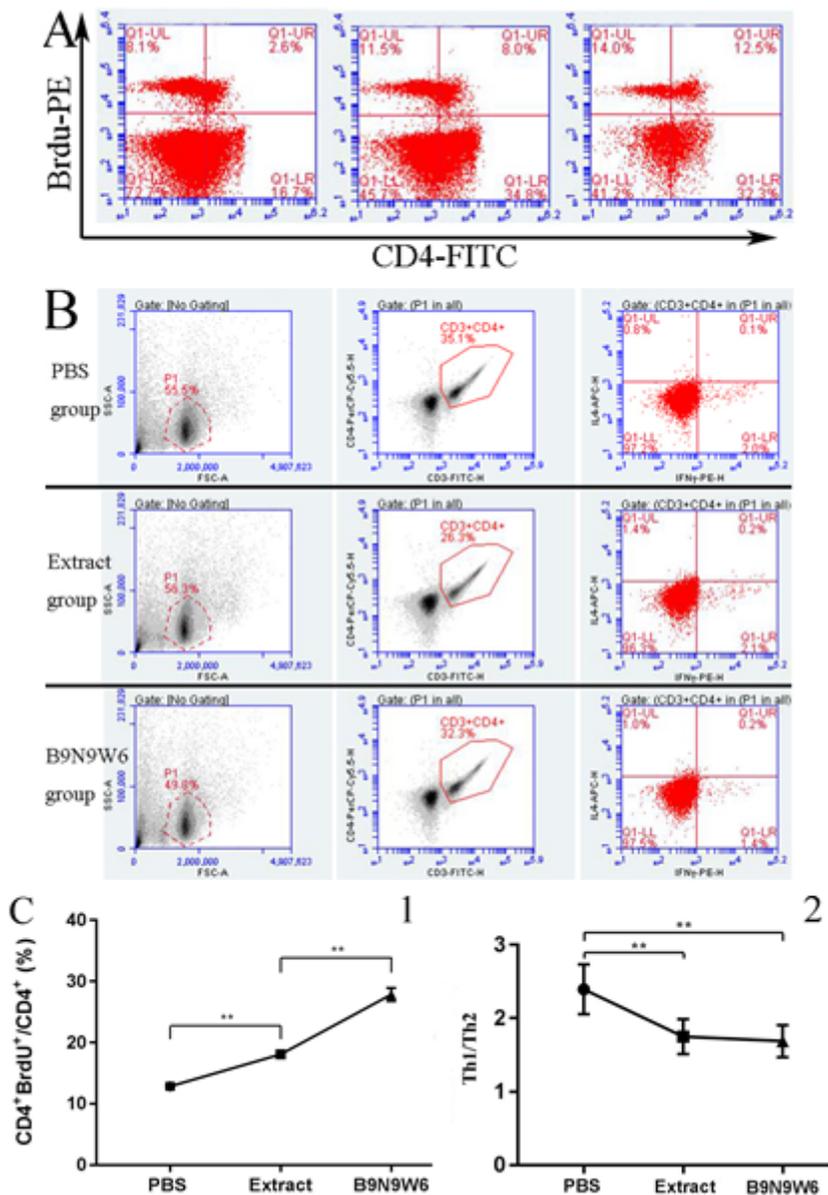


Figure 10

Proliferation of CD4⁺ T cells (A) and polarization of Th2(B) both treated with B9N9W6. C1: Proportion of CD4⁺ BrdU⁺ T cells in terms of total CD4⁺ T cells in each experimental group. C2: Ratio of Th1/Th2. Values are presented as the percentages. ** P < 0.01.

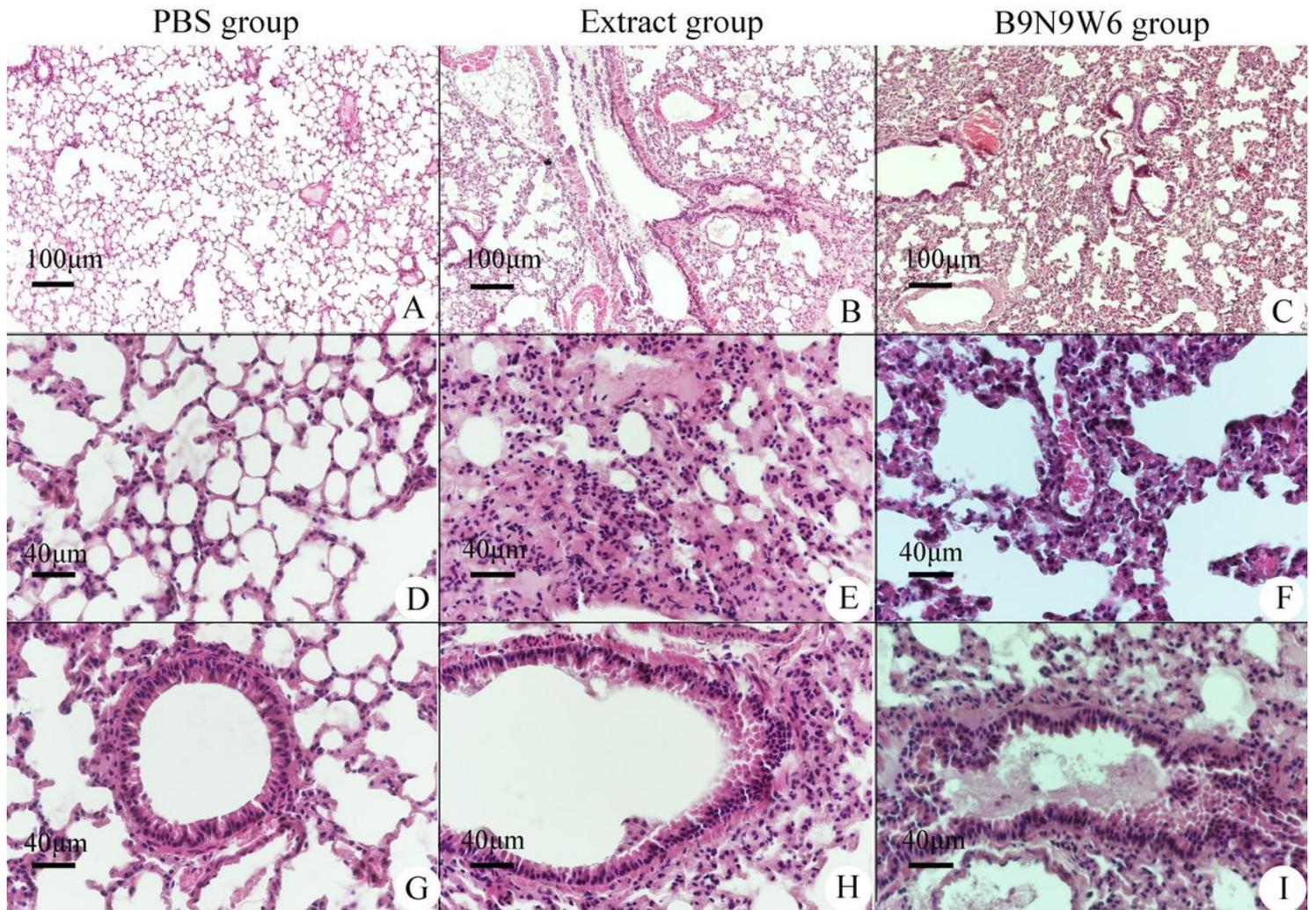


Figure 11

Inflammation and allergic pathological features in lung tissue of mouse model treated with B9N9W6. A-C: lung tissue (100×). D-F: alveolar (400×). G-I: terminal bronchus (400×).

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