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RESEARCH ARTICLE

Analysis of total proteins in pollen of *Humulus scandens* Lour in Wuhan Region of China by two-dimensional electrophoresis

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Abstract Total proteins in the pollen of *Humulus scandens* Lour, one of the most popular aeroallergens in China, were analyzed by two-dimensional electrophoresis in the current study. The proteins were extracted by Trichloracetic acid (TCA) method, and then separated by isoelectric focusing as the first dimension and SDS-PAGE as the second dimension. The spots of proteins were visualized by staining with Coomassie Brilliant Blue. After analysis with software (ImageMaster 2D), 122 different proteins were detected; isoelectric point (pI), Molecular weight (MW) and relative volume of each protein in the pollen were also discovered. This is the first high-resolution, two-dimensional protein map of the pollen of *Humulus scandens* Lour in China. Our finding has built a solid foundation for identification, characterization, gene cloning and standardization of allergenic proteins in the pollen of *Humulus scandens* Lour for further studies.

Keywords *Humulus scandens* Lour, pollen allergen, proteins, two-dimensional electrophoresis

1 Introduction

Humulus scandens Lour, an annual herbaceous plant, is mainly distributed in Wuhan Region and all across China except Qinghai and Xinjiang (Yi et al., 2001; Liu et al., 2002). During the last allergen survey in the mainland in 1995, the pollen of *Humulus scandens* was considered as an important aeorallergen in the middle and north of China. The pollen of *Humulus scandens* in copious is the main cause of seasonal allergic rhinitis (hay fever), asthma and nasal mucus in those regions (Sun et al., 2002). In recent years, research in allergy provoked by grass pollen has focused on the characterization of relevant grass pollen as allergens because as many as approximately 40% of allergic individuals start their symptoms immediately after contact with such pollens. It has been verified that allergenic proteins in pollens are a kind of proteins with molecular weight of about 30 kD, which are quickly and profusely released upon hydration (Tinghino et al., 2002; Wang et al., 2001). In this research, the proteins in the pollen of *Humulus scandens* were analyzed by two-dimensional electrophoresis, marking the first time that 2-D electrophoresis was applied in grass pollen allergen analysis (Porubleva et al., 2001; Liu et al., 2001). The results of this research will bring a valuable reference for further research on the pollen allergen of *Humulus scandens* Lour.

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2 Materials and methods

2.1 Pollen collection

The pollen of *Humulus scandens* Lour was collected during August to September; the fresh pollen was stored in −70°C before protein analysis.

2.2 Extraction of pollen protein

Proteins of the pollen were extracted according to references (Santoni et al., 1994) with the minimal change as follows. The pollen was ground in a mortar with liquid nitrogen, with the collected ground material placed in an eppendorff tube (tube weight 1.0 g). 10% w/v trichloroacetic acid and 0.07% v/v 2-mercaptoethanol were added in cold acetone. The precipitated proteins were centrifuged in microfuge for 15–20 min at 14,000 rpm. The pellet with cold acetone containing 0.07% v/v 2-mercaptoethanol (approx. 1 mL) were washed to remove pigments and lipids until the pellet was colorless. The proteins under vacuum were dried for 5–10 min. The proteins were resuspend in the appropriate rehydration buffer by extracting proteins in a water-bath for 15–30 min. The supernatant containing predominantly soluble proteins was centrifuged and collected.

2.3 Two-dimensional analysis

2.3.1 Rehydration of the immobilized pH gradient (IPG) strips

300 μL of the protein sample was piped into a special channel, the coversheet from one of the IPG strips was peeled, and the strip gel was gently placed upside down onto the sample. Each of the strips with 2 to 3 mL of mineral oil was overlaid to prevent evaporation during the rehydration process. The tray was left sitting on a level bench overnight (11–16 h) to rehydrate the IPG strips and load the protein sample.

2.3.2 Isoelectric focusing

The IPG strip was transferred to the corresponding channel in the focusing tray. Each IPG strip was covered with 2 to 3 mL of fresh mineral oil. The focusing tray was placed into the PROTEAN IEF cell. The IEF procedure is 250 V, 20 min, 10000 V, 2.5 h, 10000 V until 40000 V-hr. The strip was conserved at −70°C after IEF finished.

2.3.3 IPG equilibrations

The IPG strips were placed in equilibration buffer I (6 mol/L Urea, 2% SDS, 0.375 mol/L Tris-HCL, 20% Glycerol, 2% DTT), shaken for 10 min, and then transferred to equilibration buffer II (6 mol/L Urea, 2% SDS, 0.375 M Tris-HCL, 20% Glycerol, 135 mmol/L Iodoacetamide) for another 10 min.

2.3.4 SDS-PAGE

SDS-PAGE was performed on the Mini-PROTEAN ® 3 Cell (BioRad) using discontinuous polyacrylamide gels with 12% separating and 4% stacking gel papered according to the laboratory manual of molecular cloning. 20 μL SDS-PAGE low weight standard (Sigma) was loaded along with extracted proteins. Electrophoresis was performed at a constant voltage of 80 V for 30 min and 100 V for 2.5 h. Protein bands were visualized by coomassie brilliant blue R-250 (Amresco).

2.3.5 Spots analysis

After being destained, the gel was scanned into a computer. A photo from the analysis was obtained by ImageMaster 2 (Amshamia).

3 Results and analysis

3.1 Two-dimensional gel analysis of total proteins in the pollen of *Humulus scandens* Lour

After being stained by Coomassie blue and destained, 122 individual protein spots were detected (PH 4.0–7.0; MW 11.012–97 kDa). Compared with traditional SDS-PAGE analysis (Burstin et al., 1993), more than 5–10 times individual proteins were detected according to the different isoelectric points (pI) and molecular weights (MW) (Fig. 1).

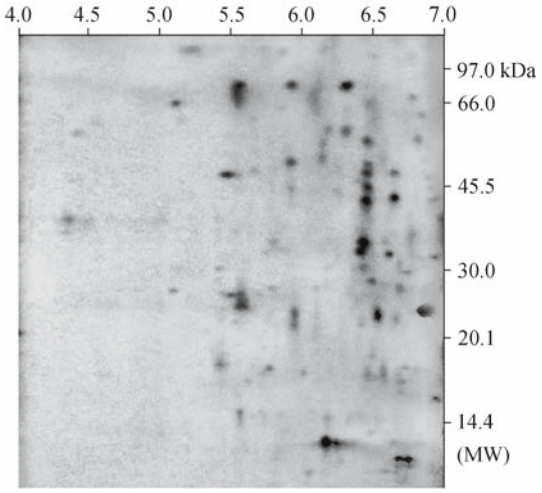

Fig. 1 The 2D map of the total proteins in the pollen of *Humulus scandens* Lour

3.2 Distribution analysis of the pollen proteins of *Humulus scandens* Lour

By use of ImageMaster 2D, the analysis showed that in the pI range of 4.0–7.0, and MW range of 11.012–94.917 kDa, the highest MW of protein spots was 94.9 kDa, and the lowest was 11.012 kDa. The MW of proteins in the 10–20 kDa range had the largest portion at 31.1% of the total number of proteins; proteins with MW in the 20–30 kDa range were next at 25.4%. Those two portions account for almost half of the protein varieties (Table 1, Fig. 2). There are 63 different proteins with pI ranging from 6.0–7.0, taking up 51.6% of all the proteins (Table 1, Fig. 3).

3.3 Analysis of the volume of different proteins in the pollen of *Humulus scandens* Lour

The relative volume of each protein spot was also detected by software (ImageMaster 2D). The analysis shows that the highest volume of the individual protein is No. 116 (pI: 6.209, MW: 12.038 kDa) (Fig. 1); the lowest is No. 62, with the volume at only 1/465 of No. 116 (Table 2).

3.4 Correlation analysis on pI and MW of different proteins

The correlation between pI and MW of different proteins in the pollen of *Humulus scandens* Lour was analyzed by SPSS (Spearman's). The results show that the coefficient between pI and MW of different proteins is −0.048 ($p = 0.603$), indicating no significant correlation between pI and MW in all the pollen proteins of *Humulus scandens* Lour.

Table 1 Analysis of 2D map of the total proteins in the pollen of *Humulus scandens* Lour

No.	pI	MW (kDa)	No.	pI	MW (kDa)	No.	pI	MW (kDa)
1	5.206	94.917	42	6.458	36.796	83	4.551	20.752
2	6.162	95.764	43	5.918	39.660	84	6.536	20.093
3	5.726	95.764	44	6.251	38.881	85	6.105	20.029
4	5.861	94.078	45	6.661	37.525	86	6.677	19.713
5	6.510	91.610	46	4.993	33.403	87	6.417	19.223
6	6.775	90.402	47	5.398	33.789	88	5.528	19.405
7	4.416	78.881	48	4.328	33.149	89	6.095	18.636
8	5.923	75.514	49	4.624	33.531	90	5.731	17.336
9	6.318	75.186	50	4.468	32.523	91	5.357	17.133
10	5.544	72.303	51	6.786	30.278	92	6.510	16.788
11	4.801	73.889	52	5.518	30.965	93	5.414	16.177
12	4.847	72.933	53	4.307	30.734	94	4.821	16.222
13	6.079	69.243	54	6.432	28.644	95	5.757	15.775
14	6.105	64.639	55	6.594	29.610	96	6.578	15.645
15	6.786	69.542	56	5.798	29.068	97	6.531	15.266
16	5.097	67.185	57	5.871	28.023	98	6.739	15.952
17	6.490	66.610	58	6.770	28.435	99	6.469	15.475
18	6.516	60.890	59	6.625	27.419	100	6.006	15.559
19	4.333	65.757	60	6.791	27.518	101	6.817	15.688
20	5.850	65.476	61	5.741	27.518	102	4.619	15.517
21	6.853	62.466	62	4.343	27.025	103	4.572	15.390
22	4.520	60.119	63	6.921	26.928	104	5.050	15.266
23	5.648	60.890	64	6.692	25.890	105	5.611	15.062
24	5.144	60.119	65	6.749	24.905	106	6.723	14.982
25	6.318	56.423	66	6.188	25.526	107	5.554	13.239
26	6.183	56.901	67	5.409	25.436	108	5.715	13.239
27	4.395	55.715	68	6.438	25.346	109	6.406	13.271
28	6.827	53.428	69	5.107	25.346	110	6.183	13.114
29	6.469	52.762	70	6.500	23.805	111	6.339	13.053
30	6.141	52.324	71	6.703	22.849	112	4.364	13.022
31	6.141	47.769	72	5.788	22.849	113	6.542	12.991
32	6.583	47.769	73	6.105	22.926	114	4.993	12.412
33	5.918	46.608	74	5.081	22.695	115	6.495	12.495
34	6.464	44.383	75	5.554	21.442	116	6.209	12.038
35	5.658	44.203	76	4.536	21.442	117	6.443	11.835
36	6.661	43.492	77	4.567	21.093	118	6.651	12.064
37	5.466	43.142	78	4.634	21.162	119	5.824	11.712
38	4.795	44.203	79	6.391	21.442	120	5.814	11.498
39	6.895	42.452	80	4.988	20.752	121	6.734	11.406
40	4.375	41.774	81	6.853	20.485	122	6.048	11.012
41	6.464	39.977	82	5.944	19.713			

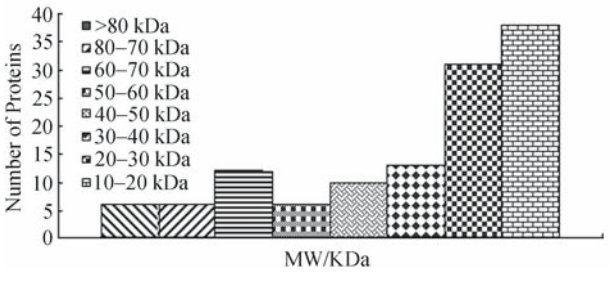

Fig. 2 MW distribution of proteins in the pollen of *Humulus scandens* Lour

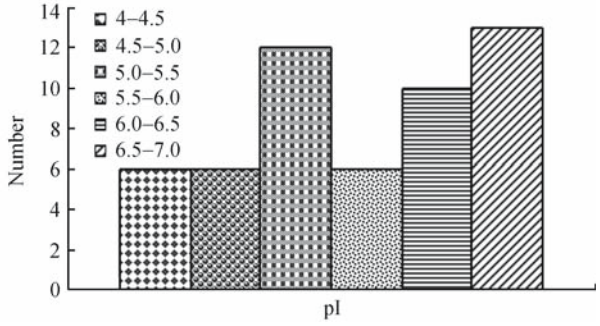

Fig. 3 pI distrubtion of proteins in the pollen of *Humulus scandens* Lour

4 Discussion

Two-dimensional gel electrophoresis (2DGE) is a method of protein separation, by which proteins in a mixture are separated according to their isoelectric point (pI) in the horizontal direction and molecular weight in the vertical direction. Along with the improvement of instruments and analytic software, the result of 2DGE will be more and more exact and

Table 2 Volume of different proteins in the pollen of *Humulus scandens* Lour

No.	Volume	No.	Volume	No.	Volume	No.	Volume
1	55,852.000	32	4,916.000	63	3,378.000	94	529.000
2	16,963.000	33	37,343.000	64	12,179.000	95	27,254.000
3	6,822.000	34	55,294.000	65	26,213.000	96	33,949.000
4	22,107.000	35	7,573.000	66	5,866.000	97	4,469.000
5	11,387.000	36	34,326.000	67	6,650.000	98	11,816.000
6	12,995.000	37	32,460.000	68	18,489.000	99	26,932.000
7	1,834.000	38	607.000	69	1,237.000	100	15,824.000
8	43,649.000	39	6,526.000	70	21,564.000	101	3,896.000
9	59,630.000	40	1,817.000	71	29,734.000	102	489.000
10	108,681.000	41	45,774.000	72	8,071.000	103	1,350.000
11	3,584.000	42	70,567.000	73	8,372.000	104	621.000
12	2,844.000	43	13,467.000	74	8,280.000	105	6,459.000
13	29,256.000	44	8,960.000	75	79,920.000	106	8,550.000
14	8,045.000	45	60,426.000	76	1,551.000	107	33,752.000
15	28,907.000	46	4,650.000	77	1,405.000	108	9,404.000
16	27,815.000	47	1,442.000	78	5,550.000	109	10,802.000
17	38,960.000	48	15,295.000	79	3,836.000	110	15,891.000
18	4,114.000	49	847.000	80	8,823.000	111	6,970.000
19	2,253.000	50	5,498.000	81	59,334.000	112	506.000
20	3,618.000	51	37,736.000	82	72,917.000	113	2,660.000
21	17,546.000	52	775.000	83	561.000	114	3,642.000
22	6,628.000	53	1,774.000	84	71,891.000	115	5,822.000
23	3,787.000	54	107,066.000	85	4,964.000	116	131,632.000
24	3,033.000	55	2,393.000	86	22,698.000	117	80,395.000
25	34,887.000	56	12,013.000	87	11,514.000	118	13,089.000
26	10,900.000	57	4,457.000	88	1,434.000	119	3,741.000
27	9,765.000	58	2,499.000	89	4,751.000	120	3,232.000
28	43,412.000	59	36,488.000	90	2,106.000	121	82,238.000
29	38,641.000	60	4,067.000	91	1,371.000	122	11,151.000
30	8,158.000	61	2,579.000	92	12,434.000		
31	20,873.0006	62	283.000	93	39,554.000		

external (Andersson et al., 2003; Zhao et al., 2000). It will play a very important role in medical research and related areas and have further applications in the future (Jia et al, 2001).

The high-resolution map obtained in this research is the first one about the total proteins in the pollen of *Humulus scandens* Lour in China. By combining with immunological hybridization, allergenic proteins will be detected by use of a specific allergenic patient's serum (Tao et al., 2004). The sequence analysis of those allergenic proteins will be a solid foundation for heterologous expression and further application in allergen specific immunotherapy (SIT).

The statistical analysis shows that there is no significant correlation between pI and MW of *Humulus scandens* Lour pollen proteins. The distribution of those proteins is random, i.e. there is no specific pI and MW distribution range of pollen proteins. In previous reports, most allergenic proteins in the pollen have MW about 30 kDa, and pI ranged in 5.0–6.0 (Poltronieri et al., 2002). Further research is needed for a different kind of plant and region prior to approval.

Allergen-specific immunotherapy (SIT) represents one of the few curative approaches toward type I hyperresponsiveness (Durham et al., 1999). However, there are three major problems associated with SIT. First, SIT is presently performed with natural allergen extracts, containing mixtures of allergens, non-allergenic and/or toxic proteins, and other macromolecules, which are hard to standardize. Second, systemic administration of allergens can cause severe IgE-mediated side effects during treatment. Finally, therapeutical effectiveness could not often be achieved because of non-standardized extracts or side effects (Ferrreira et al., 2002). With the clarification of the nature, sequence and three-dimensional structure of several important allergens, molecular level reorganization of allergens and IgE antibodies will become available.

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