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Disclosure of the Tuberous Lectin Composed of Homogeneous Tetramers in *Pinellia pedatisecda* Schott

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Abstract *Pinellia pedatisecta* (Schott) and *Pinellia ternata* (Thumb) Breit, whose tuberous stems are an important Chinese medicine, taxonomically belong to Pinellia, Araceae species. Pinellia contains various lectins in their tubers, leading to distinct roles in Chinese medicine. Difference of the lectins, however, is little known between *P. pedatisecta* and *P. ternata* tubers. For addressing to this purpose, lectins were isolated from their tuberous stems, purified through porcine thyroglobulin chromatography, analyzed with 2D-gel and Q-Trap mass spectrometry, and evaluated with hemagglutinating assays. The results showed that they possess completely different components of lectins though the lectins could specifically bind to mannose. *P. ternata* had the tuberous lectin composed of heterogeneous tetramer $(L1)_2(L2)_2$ with the similar molecular weight but distinct pI 5.8 and pI 6.2. Comparatively, *P. pedatisecta* mainly contained the tuberous lectin composed of homogeneous tetramer with the same molecular weight and pI 5.8. As a result of the lectin difference between *P. pedatisecta* and *P. ternata*, it probably leads to distinct pharmacologic variability. From this perspective, *P. pedatisecta* could be useful for anticancer research in some ways.

Keywords *Pinellia pedatisecta* · *Pinellia ternata* · Tuberous lectins · Homogeneous tetramer · Heterogeneous tetramer

Pinellia ternata and *Pinellia pedatisecta*, whose tuberous stems are important traditional medicines used in China widely, are classified into Pinellia, Araceae species [1, 2]. A variety of bioactive components existed in the tuberous stems [3, 4]. Among them,

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P. ternata could knock the prophase pregnancy and promote mitogenic activity to lymphocyte [5, 6], and *P. pedatisecta* shows notable antitumor efficacy [7, 8]. As compared with other medicine herbs, it is the specific lectins in their tubers that render the two distinctive.

Lectins, multivalent cell-agglutinating proteins, with the specific sugar-binding activity, play diversely important roles in biological organisms. Up until now, the Pinellia lectin is the only one discovered from plants, which could specifically bind with mannose but not with glucose [5], and it played a wonderful role in the theoretical and applied studies.

Pinellia lectin protein was first purified in 1993. It was extracted from the fresh tuber of *P. ternata*, precipitated with ammonium sulfate at 95% saturation, run through affinity chromatography with porcine thyroglobulin and then eluted by water. It revealed 12 kD more or less protein as well as some hemagglutinating activity toward rabbit erythrocytes. It was finally named *P. ternata* lectin (PTL) [9]. The other lectin protein from *P. pedatisecta* has been separated with gel filtration or hydrophobic interaction chromatography on Sephadex G-100 column, ion exchange chromatography on DB-32 column, and electrophoresis. It exhibited the similar protein and was named *P. pedatisecta* lectin (PPL) [10]. Yet, the two lectin proteins are not exactly the same. The full length cDNA of PTL derived from the inflorescence of *P. ternata* has been recently cloned using rapid amplification of cdna ends-polymerase chain reaction [11]. It contained 1,191 bp, one 269aa open reading frame (ORF), and 25aa signal peptide. The matured protein included 245aa with the predicted 26 kD and pI 6.07. Furthermore, it had three mannose-binding boxes. Also, transgenic tobacco expressing PTL gene has been reported to enhance resistance to aphids [12]. However, PPL has been little known about those characters, especially its mannose-binding boxes. According to their distinctive medicine action, there should be some crucial differentiation between PTL and PPL. To further understand the medicine functions of both *P. ternata* and *P. pedatisecta*, here we reported that main differences of PTL and PPL through affinity chromatography, 2D-PAG, Q-Trap MS, and hemagglutinating activity assay, which could be crucial for PPL pharmacologic variability.

Materials and Methods

Plant Materials

P. pedatisecta was collected from Tian-Mu Mountain, Zhejiang Province, and *P. ternata* from Xinjiang City, Shanxi Province. Then, they were parallel cultivated in the greenhouse for more than two generations. Their fresh tubers were used for the following assays.

Soluble Protein Extraction

Five grams of the peeled tuberous stems were taken, cut into small pieces, transferred to a chilled mortar, ground into homogenate with 20 ml, 0.145 M NaCl, and centrifuged at 2,000 rpm, 4 °C for 15 min. Then, the supernatant was taken into another centrifuge tube, added with a powder of $(NH_4)_2SO_4$ at the final concentration of 54% (*w*/*v*), vibrated immediately until the $(NH_4)_2SO_4$ dissolved completely, and centrifuged again at 10,000 rpm, 4 °C for 15 min. The precipitant was left, and dissolved with 2 ml, 0.145 M NaCl, or buffer A (50 mM pH 7.8 Tris-HCl, 1.0 M NaCl), and then exhaustively dialyzed against the distilled Milli-Q water or buffer A at 4 °C for 48 h, respectively. The dialyzed sample was centrifuged at 12,000 rpm, at 4 °C for 15 min, then the debris was removed,

and the water-dialyzed sample was lyophilized into a crude powder and stored at 4 °C, and the buffer A-dialyzed sample was directly used for the following affinity chromatography assays. In addition, the water used was fully deionized (>18.2MΩ) with a Milli-Q system (Millipore SAS 67120 MOISHEIM, France).

Isolation of Lectins

As described by Wang [9], the lectin was isolated through the porcine thyroglobulin affinity chromatography. Firstly, 5.0 ml porcine thyroglobulin cross-linked 4% beaded agarose (Sigma) was loaded into a column (10×100 mm), settled down naturally, and equilibrated with 50 ml buffer A. Consequently, 0.5 ml dialyzed sample (3.5 mg protein/ml) was added onto the column and flowed slowly. The column was initially washed with 150 ml buffer A completely to remove the non-linked elements, as followed by Milli-Q water to elute lectin. The fractions were collected and measured in absorbance at 280 nm. When the absorbance was down to almost zero after the first peak appeared, the eluting solution of 8.0 M urea instead of Milli-Q water and continued to elute different lectins. Both the eluted fractions were captured per 1.0 ml. The fractions were gathered under the same peak, quantified with Bradford method, lyophilized into powder, and stocked at −40 °C.

Hemagglutinating Activity Assay

Based on the previous methods [5], hemagglutinating activity (HA) assays were investigated for the separated lectins. Fifty microliter series of them with the doubled dilutions was prepared into microtiter U-plates and mixed 50 μl of the 2% rabbit erythrocytes suspension in physiological saline (0.145 M NaCl) at room temperature (20 °C), which was prepared as usual. The results were recorded after about 1 h when the blank had fully sedimented under a microscopy. Hemagglutination unit (HU) was expressed as the reciprocal of the highest dilution showing detectable hemagglutination and the specific activity (HU/mg) was calculated.

pI Values Assay

According to the protocol described in the previous report [13], 200 μl 0.5 mg/ml lectin was gently mixed with 200 μl 0.05 M phosphate-buffered saline buffer with serial pH values ranging from 4.8 to 8.2 by 0.1 intervals, left for 15 min, centrifuge at 5,000 rpm for 15 min. Then, the supernatants were measured at 280 nm. The pH value was defined as pI value where the absorbance value declined drastically.

Mass Spectrometry

Q-TRAP™ LC–MS/MS system (Applied Biosystems, USA) was used for an assay. The columned sample above was dissolved within 80% acetonitrile (high performance liquid chromatography grade, Fisher Scientific products) containing 0.01% trifluoroacetic acid, and its final concentration was 100 pg/ul. A volume of the 100 μl solution was sampled with 3 μl/min syringe pump flow rate under the conditions: Ion Source of Turbospray™ interface and scan of enhanced multi-charged with the positive ion modes, and the optimized parameters: curtain gas at 10, ion source gas 1 at 20, collision gas at high, ionspray voltage at 5,500 V, declustering potential at 30 V, entrance potential at 10.0 V, collision energy at 10.0 eV and m/z scan spectrum from 200 to 1,700 amu.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out as described by O'farrell [14] yet with some modifications. Iso-electric focusing (IEF) gel was freshly mixed with 0.54 g urea, 260 μl distilled water, 14 μl pH 3.5~9.5, 26 μl pH 5~8 ampholines, 200 μl 30% polyacrylamide, and 200 μl Triton-100A. After completely dissolved, the mixture was added with 3 μl TEMED and 10 μl 10% ammonium persulfate. Immediately, 200 μl of the mixture was taken and mixed with 2.5 ul, 0.04 ug/μl of the columned lectin sample. Consequently, it was injected into a glass tuber (1.5 mm×100 mm), and polymerized at 28 °C for 1 h. The IEF gel was run at 100 V for 30 min, and then upraised the voltage with 100 V per 30 min. When reached to 600 V, it was constant for overnight. Before being stopped, it was run at 800 V for 2 h. As followed by the IEF electrophoresis, each gel was immersed in 5 ml equilibrating buffer (50 mM pH 6.8 Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 20 mM DTT) for 30 min. Then it was transferred onto the top of 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel. It was run at 200 V for 2 h. Finally, the gel was stained with 0.05% Coomassie Brilliant Blue R-250. Its images were captured with a flatbed scanner of Bio Imaging Systems (GeneSnap from SynGene, Gene Genius, Gene Co., Ltd., Internal, Cambridge CB4 1TF, UK).

Results

Different Ratio of the Water-eluted Lectin (WEL) to the Urea-eluted Lectin (UEL) Between *P. ternata* and *Pinellia pedatisecda*

It is based on the molecular weight, hardly to isolate and purify the lectins from the tubers of *Pinellia* because they have a very similar molecular weight, around 12 kD, to the other proteins. According to the previous reports that the *Pinellia* lectins had a mannose-binding specificity, the affinity chromatography with the porcine thyroglobulin cross-linked 4% beaded sepharose was used to address it. The result showed two sharper peaks, one eluted with water and the other with 8 M Urea (Fig. 1). It also showed a dramatic difference in the ratio of WEL to UEL between *P. ternata* and *P. pedatisecta* (Table 1). They were recovered as the WEL and the UEL, respectively.

Distinct Hemagglutinating Activity of the WEL and UEL to Rabbit Erythrocytes

HA of the WEL and UEL lectins were investigated by rabbit erythrocytes in both *P. ternata* and *P. pedatisecta*. The result from *P. ternata* showed that the HA of the WEL was higher than that of the UEL. On the contrary, the WEL represented lower HA than the UEL in *P. pedatisecta*. Meanwhile, the highest HA to rabbit erythrocytes was observed in the WEL of *P. ternata* and the UEL of *P. pedatisecta* (Table 2).

Various Proteins Detected in *P. ternata* and *P. pedatisecta*

For further understanding the ratio difference between *P. ternata* and *P. pedatisecta*, a mass spectrometry analysis of the lectins was carried out. Based on the EMS scan for the lectins, electrons clusters were shown mainly to accumulate m/z ranged from 700 to 1,300. The result showed that there were four proteins with the molecular weight of 12,062 Da, 12,659 Da, 24,122 Da, and 25,288 Da in the WEL of *P. ternata* (Fig. 2a). Interestingly, the

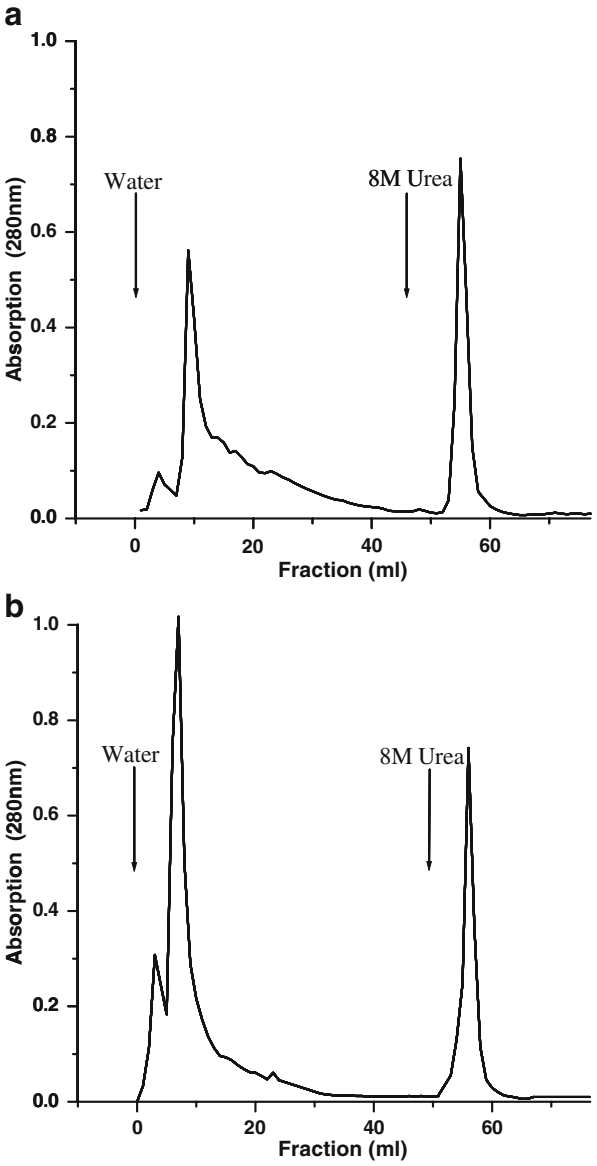

Fig. 1 Affinity chromatography for lectins of *P. ternata* (Thumb.) Breit. and *P. pedatisecta* Schott. Porcine thyroglobulin immobilized on cross-linked 4% beaded agarose column (1×10 cm) was used for this isolation. Fractions were monitored in 1.0 ml each at 280 nm. The former peak was produced by water eluting, and the latter by 8 M urea eluting (*arrows*). It showed that the water-eluting lectins (WEL) had a higher content in the tuberous root of *P. pedatisecta* (**b**) than that of *P. ternata* (**a**) though the urea-eluting lectins (UEL) are equal

former two proteins were approximately half of the latter ones, respectively. In the UEL, two proteins were also observed with the molecular weight of 12,068 Da and 12,643 Da, very similar to the corresponding 12,062 Da and 12,659 Da proteins of the WEL (Fig. 3a). On the contrary, the WEL of *P. pedatisecta* clearly represented 12,074 Da and 24,147 Da proteins (Fig. 2b), and the UEL had 11,905 Da and 23,810 Da. proteins, which the former was a half of the latter in the molecular weight (Fig. 3b). Noticeably, the proteins represented in the WEL seem to be slightly different from that in the UEL.

Table 1 Comparison of the ratios of the WEL to UEL lectins between *Pinellia ternata* and *Pinellia pedatisecta*.

Sample	Total protein (mg)	WEL (mg)	UEL (mg)	WEL/UEL	Lectin/total protein (%)
P. pedatisecta	3.52	0.273±0.011	0.130±0.009	2.10	0.114
P. ternata	3.52	0.177±0.013	0.175±0.006	1.01	0.100

Two Proteins Observed in the WEL and UEL Mixture of *P. ternata* and One in that of *P. pedatisecta*

For further understanding characters of the diverse lectins, it was carried out with two-dimensional gel electrophoresis. As a result of the WEL and UEL mixture of *P. ternata*, there were two spots on the 2D-gel with their molecular weight of about 12 kD, but located at different pI value (Fig. 4a). One could be raised from 12,062 Da lectin, and the other at pI 6.2 from 12,659 Da. But, the WEL and UEL mixture of *P. pedatisecta* only led to one spot presenting on the 2D-gel with their molecular weight of about 12 kD and pI 5.8 (Fig. 4b), indicating that 11,905 Da lectin containing in the UEL was similar to 12,074 Da one in the WEL. The result was well shared with MS analysis.

Discussion

Both *P. ternata* and *P. pedatisecta,* belonging to Araceae species, whose tubers are widely used as a traditional Chinese herb medicine, shared similar botanic characters. However, many reports have indicated that they have quite distinct medical functions [6, 8, 15]. Obviously, those differences could be related to the components of their tubers, especially the chief elements including polysaccharide, alkaloids, amino acids, peptides, and proteins. As known to us, the most characteristic element related to their medical function is *Pinellia* proteins, especially lectins [5]. Therefore, it could be very useful to discover more roles in the traditional Chinese medicines if differences of the lectins are better understanding between the two similar tubers. In the previous researches, little attention was paid to the differences of the lectin between *P. ternata* and *P. pedatisecta*. Here, we have found some significant differences in the lectins derived from them using distinct methods.

There was a quantitative difference in ratio of the WEL to the UEL between *P. ternata* and *P. pedatisecta* (Table 1). Usually, plant lectins could be eluted with water on

Table 2 Evaluation of the WEL and UEL lectins derived from *Pinellia ternata* and *Pinellia pedatisecta* in hemaggulutinating activity toward the rabbit erythrocyte.

Samples	Concent.* (ug/ml)	2^{-3}	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
WEL (*P. ternata*)	0.2	+++	+++	+++	++	++	++	+	−
UEL (*P. ternata*)	0.39	+++	+++	+++	+++	++	+	−	−
WEL (*P. pedatisecta*)	0.78	+++	+++	++	++	+	−	−	−
UEL (*P. pedatisecta*)	0.2	+++	+++	+++	+++	++	++	+	−
Total protein (*P. pedatisecta*)	1.56	+++	++	++	++	−	−	−	−
Total protein (*P. ternata*)	1.56	+++	++	+	+	−	−	−	−

0.1 mg/ml as the initial concentration (2^0)

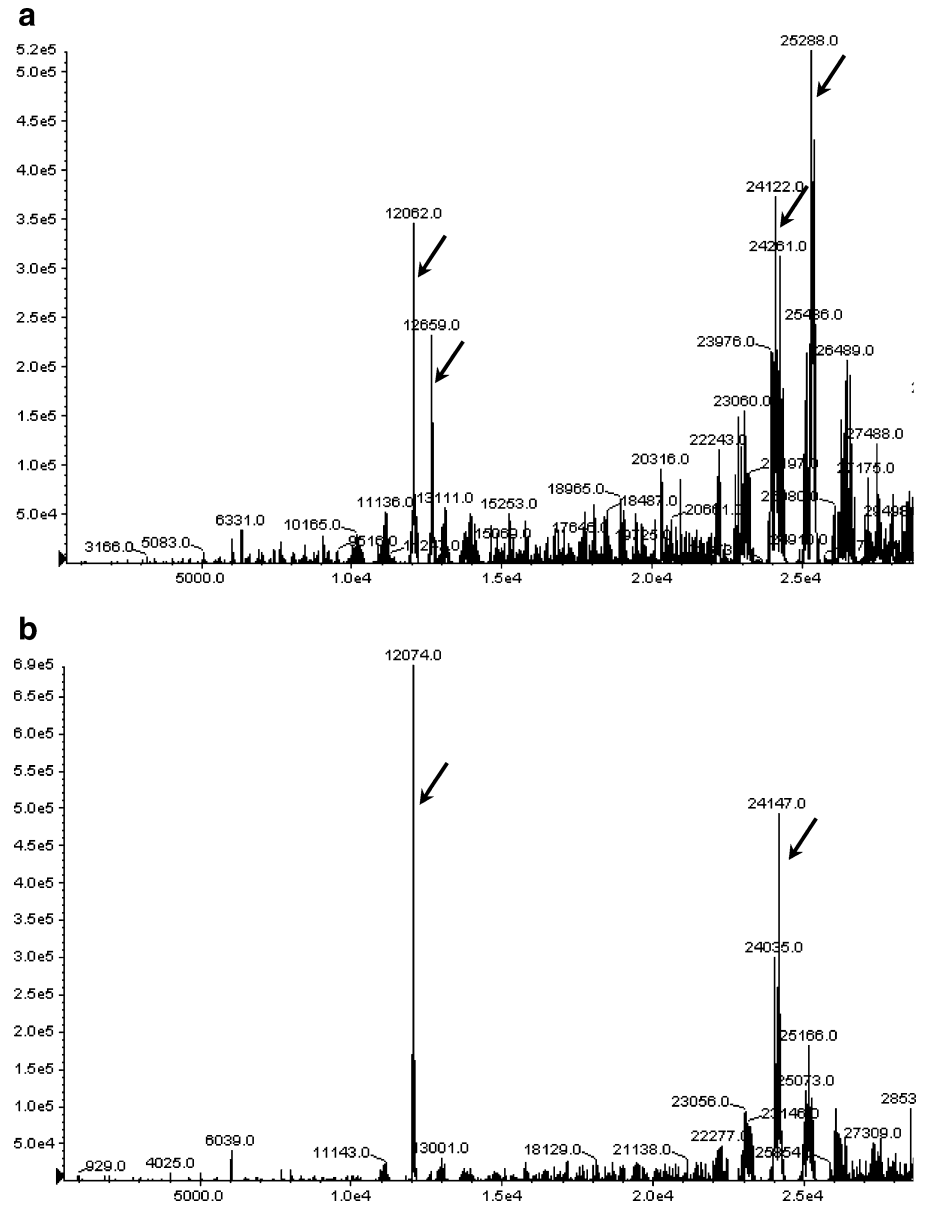

Fig. 2 EMS spectra of the WEL of *P. ternata* (Thumb.) Breit. and *P. pedatisecta* Schott. Four dominant proteins were showed in the WEL of *P. ternata* with the molecular weight of 12,062 Da at monomer referred to 24,122 Da at its dimer, and 12,659 Da referred to 25,288 Da at the dimer (**a**). Whereas, only two proteins presented in the WEL of *P. pedatisecta* (**b**) with the molecular weight of 12,074 Da at monomer and 24,147 Da at its dimer

chromatographic column of the porcine thyroglobulin, but we tried to elute with 8 M urea after water elution and have obtained the other kind of proteins. This protein has been approved to have hemagglutinating activity to rabbit erythrocytes (Table 2). Obviously, this quantitative difference could be not only the superficial phenomenon but the essence

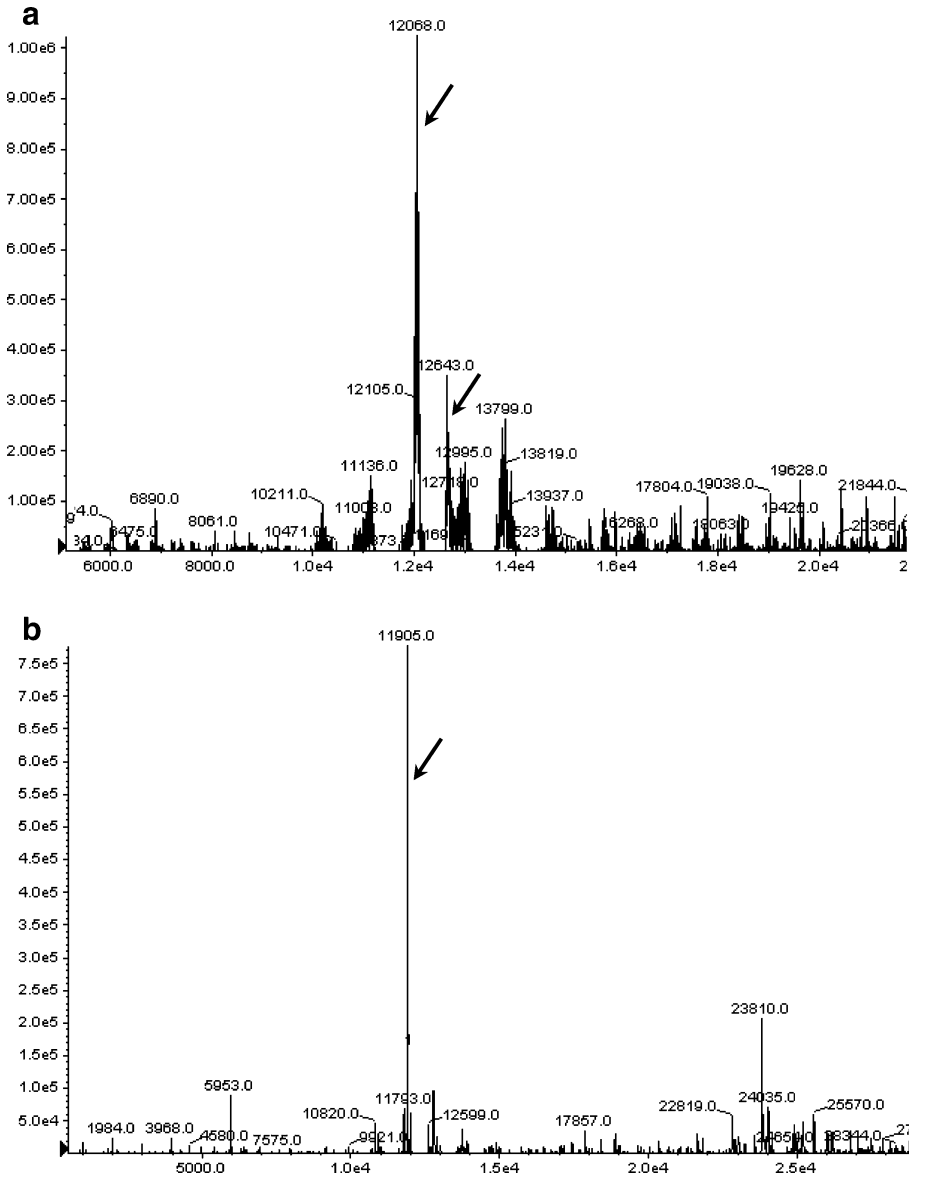

Fig. 3 EMS spectra of the UEL of *P. ternata* (Thumb.) Breit. and *P. pedatisecta* Schott. Two dominant peaks were observed on their m/z, indicating that two dominant proteins contained in the UEL of *P. ternata*. Their molecular weight, 12,068 Da and 12,643 Da, was similar to the 12,062 Da and 12,659 Da detected in the WEL, respectively. In the UEL of *P. pedatisecta*, it showed two proteins with molecular weight of 11,905 Da at monomer and 23,810 Da at its dimer

including hemagglutinating activity, which may be related to their pharmacologic variability. The result from hemagglutinating activity assays showed distinct hemagglutinating activity of the WEL and UEL to rabbit erythrocytes from *P. ternata* to *P. pedatisecta* (Table 2), suggesting that they contain diverse lectins. Also, distinct hemagglutinating activity might be raised from conformational isomerism caused by different elution between the WEL and

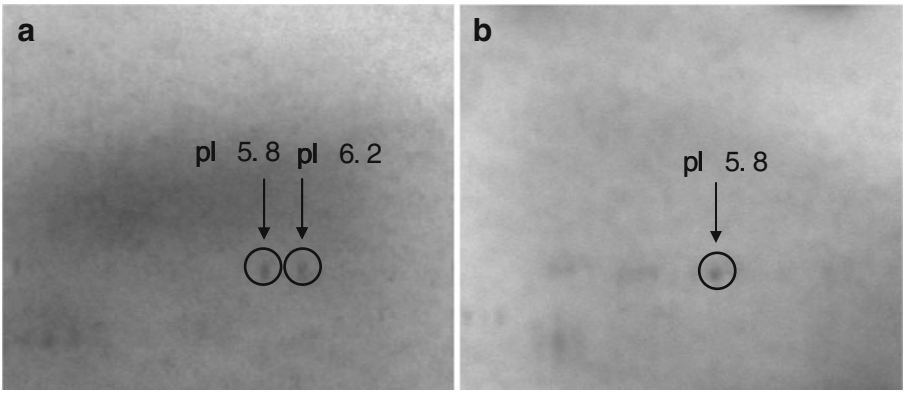

Fig. 4 2D-PAGE for the mixture of the WEL and UEL samples of *P. pedatisecta* Schott as compared to that of *P. ternata* (Thumb.) Breit. The sample was prepared with the mixture of 0.1 μg WEL and 0.1 μg UEL. The first dimension was carried out with 6% gel, pH gradient 3.5~9.5, and 9 M urea. The second dimension was run on 15% SDS-PAGE. The gel was stained with 0.01% Coomassie Brilliant Blue G-250. One spot (*arrow*) was illustrated at the cross of pI 5.8 and 12 kD from both *P. pedatisecta* and *P. ternata* But an extra spot appeared at the cross of pI 6.2 and 12 kD in *P. ternata*

UEL of *P. pedatisecta* though they are in homogeneous tetramers (see below). These needs to be further clarified.

The WEL and UEL have been analyzed with mass spectrometry, and various proteins have been detected from *P. ternata* to *P. pedatisecta*. As well as it is known that the natural lectin derived from Pinellia is a tetramer linked by electrovalent bond and hydrophobic power [16], it is suggested that there may be monomers, dimmers, and tetramers in their separation. In this assay, there are four proteins detected in the WEL of *P. ternata*. According to their relationship of the molecular weight of 12,062 Da, 12,659 Da, 24,122 Da, and 25,288 Da, two dominant proteins should be considered, 12,062 Da and 12,659 Da at monomer referred to 24,122 Da and 25,288 Da at dimer, respectively (Fig. 2a). Also, the proteins detected in the UEL have a similar relationship (Fig. 3a). So, It is strongly suggested that lectins from the tubers of *P. ternata* are composed of heterogeneous tetramers, likely $(L1)_2(L2)_2$. On the contrary, tuberous lectins are made up of homogeneous tetramers in *P. pedatisecta* because only two proteins have been detected in either WEL (Fig. 2b) or UEL (Fig. 3b), one at monomer and the other at dimer. Noticeably, this lectin was previously reported to consider as a heterogeneous tetramer composed of polypeptides A and polypeptides B with 43% identity though only one 771 bp ORF [17, 18]. Obviously, polypeptides A and polypeptides B with significantly difference could have been easily separated. In fact, the result from a mass spectrometry analysis showed that only one polypeptide in *P. pedatisecta*. This homogeneous tetramers have been also proved by an evidence that only one protein is observed on 2D-gel in the WEL and UEL mixture of *P. pedatisecta* (Fig. 4b), whereas two proteins in that of *P. ternata* (Fig. 4a).

This finding may be significant on not only indicating some differences in their hemagglutinating activity but better understanding on their pharmacologic variability, especially *P. pedatisecta* as an anticancer-research Chinese herb. Also, we have firstly reported that the lectin from *P. pedatisecta* recognized a fraction of acute myeloid leukemia cells, Kasumi-1, showing mannose-exposing phenotype [19]. It is worthy of further investigation on other properties such as their amino acid sequences, structural or conformational features, and physiochemical characters, especially related genes and their prokaryotic expression[20].

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