SDS-PAGE and 2-DE protein profiles of *Ganoderma lucidum* from different origins

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Abstract: *Ganoderma lucidum* (Chizhi in Chinese) is one of the most valuable and widely used medicinal fungi in traditional Chinese medicines (TCMs). Most of previous studies were focused on the triterpenoids and polysaccharides of *G. lucidum*, whereas less attention had been paid on the protein, which is another bioactive compound in it. In the present study, protein maps of fourteen *G. lucidum* samples were comprehensively analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis (2-DE). The results indicated that there were significant differences in protein profiles of *G. lucidum* samples from different origins. Furthermore, previous reported bioactive proteins from the fruiting bodies of *G. lucidum*, were mainly distributed in 4 taxa (A, B, C and D) based on their molecular weights on the 2-DE maps. The proteins should be considered as marker for the quality control of *G. lucidum*, because the proteomic variation may affect on their pharmacological activities.

Keywords: *Ganoderma lucidum*, SDS-PAGE, 2-DE, bioactive protein, protein profiles.

INTRODUCTION

Ganoderma (Lingzhi), known as "the mushroom of immortality" in China, is a basidiomycete white rot fungus used as a tonic for promoting health and longevity for more than 2000 years (Russell, 2006). Up to now, over 200 species of *Ganoderma* have been found worldwide, among them, *G. lucidum* (Chizhi in Chinese) as well as *G. sinense* (Zizhi in Chinese) are the most well-known and wildly used in China. It has been demonstrated that *G. lucidum* contains various bioactive compounds, such as triterpenoids, polysaccharides, adenosine, alkaloids and proteins, etc, (Shiao, 2003; Heleno *et al.*, 2012). *G. lucidum* has multiple pharmacological activities, including anti-epileptic (Wang *et al.*, 2014), anti-viral (Zhang *et al.*, 2014), anti-tumor (Coer *et al.*, 2014) and hepatoprotective (Liu *et al.*, 2014), as well as other therapeutic functions such as anti-angiogenic (Boh *et al.*, 2007). Nowadays, along with the wild *G. lucidum*, various products have been prepared from the cultivated fruiting bodies of *G. lucidum* and have been commercialized as dietary supplements worldwide (Lai *et al.*, 2004). Studies indicated that different origins of *G. lucidum* had significant differences in chemical components and bioactivities (Stojkovic *et al.*, 2014). However, recently published reports could not effectively identify the origins of *G. lucidum* according to the content of polysaccharide and triterpenes determined by HPLC (Sun *et al.*, 2014; Ha Thi *et al.*, 2015).

On the other hand, modern researches have demonstrated that *G. lucidum* contains a wide variety of bioactive proteins, including fungal immunomodulatory proteins (FIPs), enzymes, lectins and glycoproteins, etc. Today, kinds of bioactive proteins have been isolated from the fruiting bodies of *G. lucidum*. For example, GLL-F (MW: 16 kDa, determined by SDS-PAGE) (Kawagishi *et al.*, 1997) and hexameric (MW: 18.5 kDa, by SDS-PAGE) (Thakur *et al.*, 2007) are lectins with specific agglutination activity. FYGLn (MW: 72.9 kDa, by SDS-PAGE) (Pan *et al.*, 2015) and FYGLa (MW: 100.2 kDa, by gel permeation chromatography, GPC) (Pan *et al.*, 2014) are water-soluble protein tyrosine phosphatase-1B (PTP1B) inhibitor for anti-diabetes. Obviously, protein is another bioactive component of *G. lucidum*, and the protein profiles may be used for evaluating and differentiating *G. lucidum* samples from different origins.

In the present study, the protein profiles of the *G. lucidum* were analyzed by 2-DE, which is a common method for separation and visualization of proteins from crude extracts of tissue samples (Sarma *et al.*, 2008). The proteins of *G. lucidum* from fourteen origins were firstly separated by SDS-PAGE. Then the proteins were analyzed by 2-DE based on molecular mass (MM) and isoelectric point (pI). Finally, the protein maps of *G. lucidum* from 14 origins were carefully compared.

MATERIALS AND METHODS

Materials and apparatus

2-DE cells used for IPG strips (pH 3-10, 13 cm), 2-D clean-up kit and IPG buffer (pH 3-10) were obtained from GE Healthcare (GE Healthcare Bio-Science, Uppsala,

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Sweden). Low melting-temperature agarose, N, N-methylene-bis acrylamide, acrylamide, 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), dithiothreitol (DTT), 2-aimino-2-(Hydroxymethyl)-1,3-propanediol (Tris), glycine, urea, thiourea, Coomassie Brilliant Blue G-250, sodium sulfate (SDS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Protein marker was obtained from Microgram-Tiantai (Chengdu, China). The ampholytes (pH 3-10) were obtained from Beijing BioDee Biotechnology Co. Ltd (Beijing, China). Analytical reagent grade ethyl alcohol absolute, acetic acid, formalin, hydrochloric acid (HCl), sodium carbonate anhydrous, bromophenol blue, ethylenediaminetetraacetic acid disodium salt (EDTA·Na_2), ammonium persulphate (AP), Triton X-100 and acetic acid sodium salt trihydrate were obtained from Chengdu Kelong Chemical Works (Chengdu, China). Analytical reagent grade glycerin and acetone were purchased from Chongqing Chuandong Chemical Co. Ltd (Chongqing, China). Silver nitrate was from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

Ultra-pure water used throughout the experiment was prepared by a water purification system (DZG-303A, Ai-ke, China). High speed refrigerated centrifuge was obtained from Hunan Cence Instrument Companies (TGL-20M, Hunan, China). Spectrafuge mini centrifuge was from Haimen Kylin-Bell Lab Instrument Co. Ltd (LX-100, Jiangsu, China). The decolorization shaking table for electrophoresis gel immobilization, sensitization, staining and decolorization was from Shanghai Yarong Biochemical Works (Shanghai, China). Electrophoresis power supple-EPS 601, Image Scanner III and Ettan IPGphor 3 systems were purchased from GE Healthcare (GE Healthcare Bio-Science, Uppsala, Sweden).

Crude protein extration from G. lucidum

Fourteen fruiting bodies of *G. lucidum* samples (table 1) were collected from local pharmacies. The samples of *G. lucidum* were identified by the corresponding author and were deposited at the Department of Pharmaceutics, School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China. Samples were shatter by pulverizer and then stored at -20^{o}C before protein extraction.

The frozen fruiting body powder (0.5 g) was grounded into fine homogeneous powder in liquid nitrogen and extracted with 10mL of Tris-HCl buffer (60 mmol/L Tris was adjusted to pH 6.8 by hydrochloric acid) including 60 mmol/L Tris-HCl, pH 6.8, 20 mmol/L DTT and 0.5% Triton X -100 in the mortar grinding for half an hour. The homogenate was transferred to micro tube and centrifuged at $1.75*10^4 \times g$ for 15 min at 4^{o}C. The supernatant was collected, and purified samples were further prepared by 2D clean-up kit (including precipitant, co-precipitant, wash buffer and wash additive). Transfer about 100μg protein sample into a 1.5 mL microcentrifuge tube and the protein was successively precipitated by precipitant and co-precipitant on the ice box. After centrifuged, the protein pellet should be dispersed in the 25 μL ultra-pure water, then add 1mL pro-chilled wash buffer and 5 μL wash additive to the suspension. The suspension should be incubated at -20^{o}C for at least 30 min. After centrifugation, followed with carefully discarding the supernatant, the pellet was re-suspended in SDS-buffer or IEF sample loading solution before electrophoresis.

SDS-PAGE

Above mentioned pellet protein samples were successively dissolved in 10 μL water and 10 μL SDS loading buffer (80 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10 mmol/L DTT, 20% glycerinum and a trace amount of bromophenol blue) and heated at 100^{o}C for 10 min. Then the samples were centrifuged at $1.25*10^4 \times g$ for 1 min (4^{o}C). The denatured protein solution was loaded in each lane and run on 12% acrylamide gel. Standard protein marker was applied to each gel. Gels were fixed overnight and stained with silver-staining. Digital image of gels were obtained by using Image Scanner III.

2-DE

The purified pellet protein samples were re-suspended in 150 μL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPs, 40 mmol/L DTT and 0.25% IPG buffer) and centrifuged at $1.25*10^4 \times g$ for 1 min (4 ^{o}C). The supernatant protein of 150 μL samples were pipetted onto a cup loading in a 17 cm ceramic strip holder with hydration immobilized linear pH gradient (IPG) strips (pH 3-10, 13 cm). The IPG strips were used to rehydrate for 15 h in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mmol/L DTT, 0.5% IPG buffer and 0.1% bromophenol blue) before IEF. The first-dimensional IEF was performed at 20 ^{o}C; the IEF procedure was as follows: (i) 250 V, gradient, 0.5 h; (ii) 250 V, step and hold, 2 h; (iii) 1000 V, gradient, 1 h; (iv) 1000 V, step and hold, 2 h; (v) 8000 V, gradient, 2 h; (vi) 8000 V, step and hold, to 15000 V. After IEF, the IPG strips were equilibrated with equilibration buffer (75 mmol/L Tris-HCl, pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS and 0.1% bromophenol blue) for 30 min (the equilibration buffer added with 30 mmol/L DTT equilibrated for 15 min and then replaced by equilibration buffer added with 40 mmol/L iodoacetamide equilibrated for another 15 min). The sealing liquid of agarose (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS, 0.5% agarose and 0.06% bromophenol blue) was loaded on the 12% polyacrylamide gel and the strips were quickly transferred to the sealing liquid. As soon as the agarose sealing liquid freezing (about 15 min in 4^{o}C), SDS-PAGE was performed using 12% polyacrylamide gel at a constant 60 V for about 1 h and 120 V for 9 h. After electrophoresis, each gel was visualized with silver staining.

Images analysis and quantification of the proteins

Gels were scanned using image scanner III, and the images were analyzed by the software PDQuest and quantity one (version 4.6.2, Bio-Rad). The quantification of protein was carried out according to Bradford (Bradford, 1976) with some modifications. Briefly, 50 µL total protein was precipitated by 1.5 mL cold 10% TCA/ acetone (-20 ^{o}C) for 2 h. After centrifugation at $1.65*10^{4}$ × *g* for 10 min (4 ^{o}C), the pellet was rinsed twice by cold acetone (-20 ^{o}C). The protein pellet was dried and then solubilized in water, the quantification of proteins were measured according to the procedure of Coomassie Brilliant Blue G-250.

RESULTS

SDS-PAGE

The SDS-PAGE has been frequently used for determination of the subunit composition of a given protein and the molecular mass of proteins (Carter *et al.*, 2013). Through the Quantity one software (Bio-rad, USA) processed the SDS-PAGE images, the results indicated that the molecular weights of *G. lucidum* proteins fluctuated between 6.5-200.0 kDa as shown in fig. 1. And noteworthy that great difference can be observed in the distribution of protein bands in the fourteen *G. lucidum* samples, even the samples (i.e., GL4 and GL13 from Hainan) from the same province had great difference in proteins numbers and abundance. Only the GL6, GL7 and GL8 had the similar proteins distribution, which contained some common bands such as 16.0 kDa, 18.5 kDa and 25.8 kDa. Furthermore, the wild *G. lucidum* of GL9 with the highest protein abundance was greatly different from the other samples in the numbers of protein band. The protein bands of fourteen samples on SDS-PAGE maps varied greatly in molecular weight, abundance and numbers, which may result from the different conditions of drying and storage, habitat or cultivated ways. In order to further compare the protein profiles of those *G. lucidum* samples, 2-DE analysis was performed in the following study.

2-DE.

About 150 µg protein for each *G. lucidum* sample was loaded and analyzed by 2-DE using a pH 3-10 IPG strips in the first dimension IEF and 12% SDS-PAGE in the second dimension on the basis of one-way experiment of SDS-PAGE. 2-DE maps of fourteen *G. lucidum* samples were shown in fig. 2. Those data (protein spots, MW, pI and relative abundance) were analyzed using PDQuest software (Bio-rad, USA). From the 2-DE profiling maps, about 11 (GL11) to 54 (GL1) protein spots were detected on the maps, some of the proteins such as 16.5 kDa and 18.5 kDa were distributed in most origins (e.g. GL5, GL6, GL8 and GL13). Corresponding to SDS-PAGE maps, GL13 contained more large molecular weights protein than the GL4; the protein spots of the wild sample GL9 had a relatively higher concentration.

Based on the molecular weights of reported bioactive proteins and their corresponding protein bands distributed in the SDS-PAGE, the proteins of fourteen samples were divided into four main taxon (A, B, C and D) with 16 kDa (Kawagishi, *et al*., 1997), 18.5 kDa (Thakur, *et al*., 2007), 42 kDa (Wang, *et al*., 2004) and 67-72.9 kDa (Ye, *et al*., 2002; Pan, *et al*., 2015) in 2-DE maps. Due to the low intensity and small molecular weight, the bands of bioactive proteins with 5.76 kDa (an inhibitor for peptidylprolyl cis-trans isomerase, PPIase) (Jin, *et al*., 2004) and 15 kDa (antifugal protein) (Wang, *et al*., 2004) were not obvious on the maps. The proteins in each taxon have the same or similar molecular weights but with different pI values. The proteins in taxon A are about 16 kDa in mass and pI values within 4.23-5.00, respectively. Taxon A, which was highly abundant and included 2-8 protein spots, was present in most *G. lucidum* samples except for GL9 on the 2-DE maps. The proteins of taxon B were 18.5 kDa in mass containing 1-8 spots and presented in most samples, except for GL2 and GL14. The proteins of taxon C were about 42 kDa in mass with faintly visible spots in seven samples. The proteins of taxon D contained two molecular weights of 67 kDa and 72.9 kDa; most of this taxon protein spots were of low abundance and cannot be clearly identified.

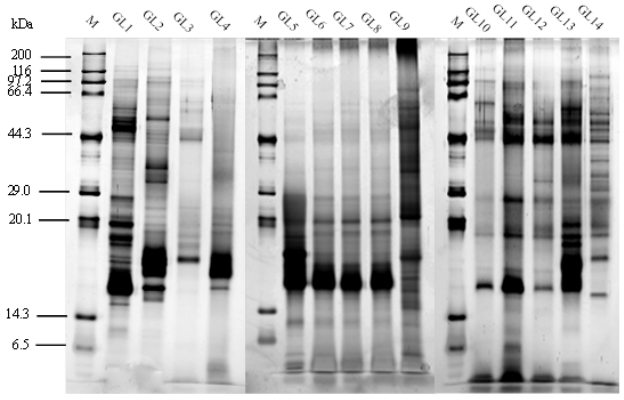


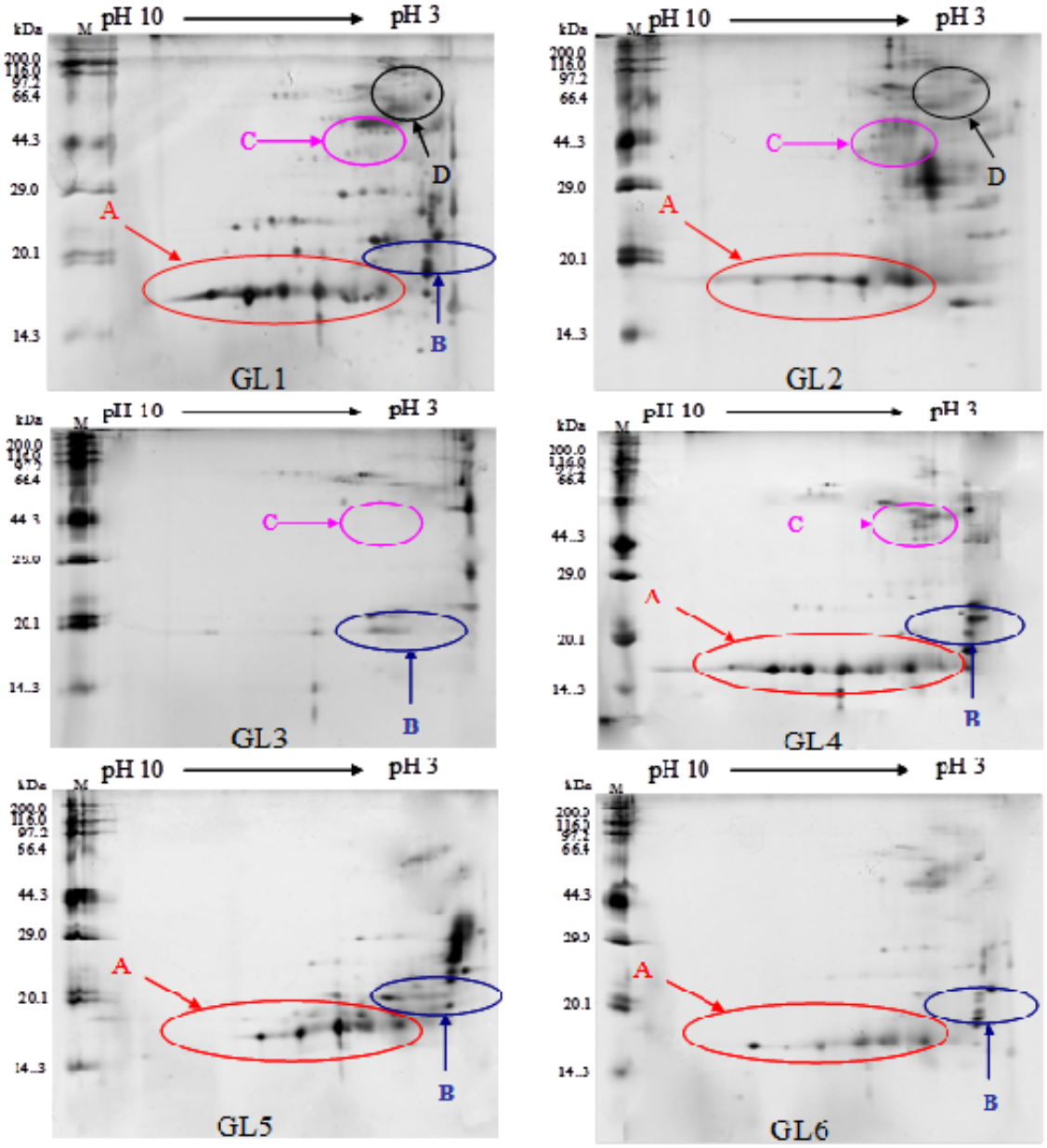
Fig. 1: SDS-PAGE protein profiles of total proteins extracted from fourteen *G. lucidum* samples. About 100 µg protein in each lane was separated by 12% SDS-PAGE gels. Molecular weight (kDa) of standard proteins (M) is shown on the left side of the figure. The 16 kDa, 18.5 kDa, and 67 kDa to 72.9 kDa proteins might be the reported bioactive proteins. Sample information for GL1 to GL14 refers to table 1.

DISCUSSION

G. lucidum is one of the most valuable and widely used TCMs. The present study is to investigate whether the protein profile approach (SDS-PAGE and 2-DE) could be used for evaluating and differentiating proteins of *G. lucidum* from different origins. In reality, the 2-DE had been successfully applied to identify different samples (Lum *et al.*, 2002; Liu *et al.*, 2012).

Table 1: Information of 14 fruiting bodies of *G. lucidum* samples

Sample code	Collecting place	Sample code	Collecting place
GL1	Shandong Province	GL8	Jilin Province
GL2	Guangxi Province	GL9	Jilin Province
GL3	Guangdong Province	GL10	Zhejiang Province
GL4	Hainan Province	GL11	Hebei Province
GL5	Yunan Province	GL12	Fujian Province
GL6	Sichuan Province	GL13	Anhui Province
GL7	Jilin Province	GL14	Hainan Province



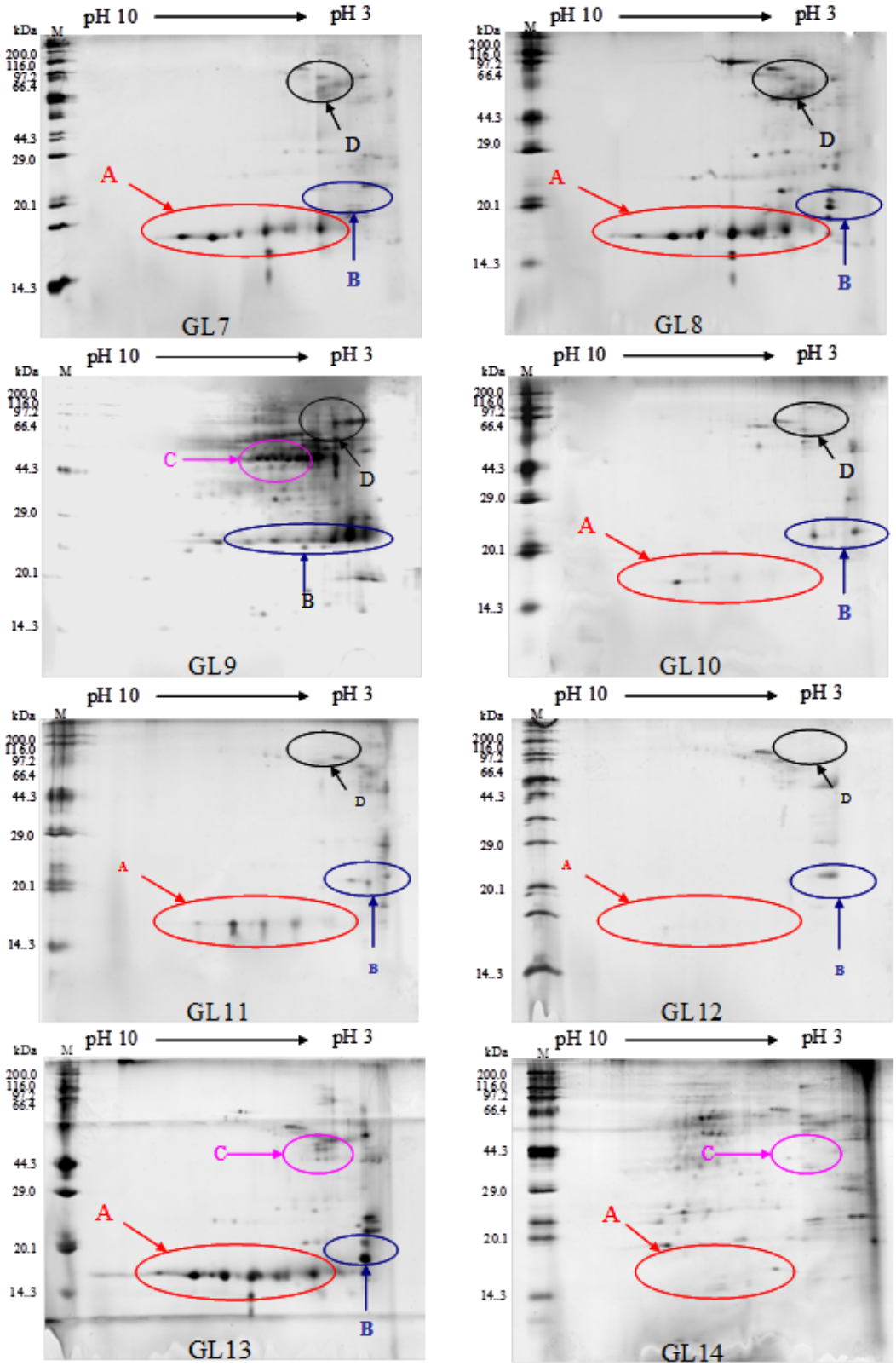


Fig. 2: The 2-DE protein maps of 14 *G. lucidum* samples. The samples were separated on 13 cm, pH 3-10 IPG strips and the 12% SDS-PAGE gels. The proteins distributed in four taxa (A, B, C and D) on the 2-DE maps were marked according to the molecular weights of reported bioactive proteins. Sample information for GL1 to GL14 refers to table 1.

The protein extraction method, which is the key step in 2-DE (Blackstock *el at*., 1999), will directly affect the electrophoretic quality, resolution and reproducibility. Plant tissues contain numerous interfering compounds including organic acids, pigments, lipids, polysaccharides, and especially for many kinds of phenolic compounds (Stalikas, 2007), which will directly affect the performance and separation in 2-DE gels. By using traditional TCA/acetone and phenol extraction methods, sugars and other substances are co-precipitated with proteins; repeated precipitation of protein by acetone leading to poor protein solubility which will seriously affect the quality of electrophoresis and loss of proteins (Wu *et al*., 2014). In the present study, the grinding and Triton X-100 gently lysis was used to extract protein and to overcome the disadvantages of extraction by SDS that can lead to vertical streaking with high background staining (Hurkman *el at*., 1986). Furthermore, the crude protein was purified by 2D clean-up kit to remove interferences in tissue.

As mentioned, there several bioactive proteins from *G. lucidum* were reported in the literatures. Combined SDS-PAGE with 2-DE maps, and based on the molecular weight of bioactive proteins mentioned in the literature, the 2-DE maps were divided into four main taxa (A-16 kDa, B-18.5 kDa, C-42 kDa and D-67 to 97.2 kDa). The proteins with 16 kDa were detected in most *G. lucidum* samples except for GL9 on the SDS-PAGE (while the existence 16 kDa were not shown in GL3 and GL9 on the 2-DE maps), meanwhile the spots of taxa A (16 kDa) in 2-DE maps were varied between 2 and 8, in which they were clearly distinguishable. The band of 18.5 kDa was also presented in most samples except for GL2 and GL14 on the SDS-PAGE, and 1 to 4 protein spots were detected in taxa B by 2-DE. The 16 kDa and 18.5 kDa proteins may be the reported lectins GLL-F (MW: 16 kDa) (Kawagishi *et al.*, 1997) and hexameric (MW: 18.5 kDa) (Thakur *et al.*, 2007) with specific agglutination activity. The protein band with 42 kDa may be a ribonuclease, which was only faintly expressed in SDS-PAGE and 2-DE maps (GL1, GL2, GL3, GL4, GL9, GL13 and GL14). The 67 kDa and 72.9 kDa proteins, which were faintly detected in SDS-PAGE and 2-DE, may be an anti-tumor protein and an anti-diabetic protein, respectively. Because of the similar molecular weights and low abundance, those proteins in taxa D were faintly distributed in GL1, GL2, GL7, GL8, GL9, GL10, GL11, GL12 and GL13. Meanwhile, the protein band of about 100.0 kDa possible contains PTPIB inhibitor for diabetes faintly existed in GL1, GL2, GL10 and GL10 on the SDS-PAGE maps but not shown on the 2-DE maps. Therefore, the results in the present study indicated that the *G. lucidum* of GL1, GL2, GL3, GL4, GL7, GL8, GL10, GL11 and GL13 may contain at least three kinds of bioactive proteins. The different intensity of band and protein spots may result from post-translational modifications and proteolysis. Moreover, the qualitative and quantitative variations may be attributed to the condition of natural variability (Natarajan, 2010), different cultivated ways (Thiellement, 2001; Bahrman *et al.*, 2005), abiotic stress (Abreu *et al.*, 2013) tolerance and water (Costa *et al.*, 1998). Therefore, the *G. lucidum* from different origins may have difference in pharmacological actions due to their great variance in protein profile.

CONCLUSION

G. lucidum of fourteen origins could be evaluated and distinguished by their protein profiles using SDS-PAGE and 2-DE. The distribution of protein bands changed greatly in SDS-PAGE maps; the proteins in taxa (A, B, C and D) are different in protein spots' numbers and abundance. In this report, many low abundance proteins had not effectively expressed in the electrophoresis maps because of the ways of extraction. The methods of PEG (polyethylene glycol) (Acquadro *et al.*, 2009; Aryal *et al.*, 2012) and CPLL (combinatorial peptide ligand libraries) (Esteve *et al*., 2013; Righetti *et al*., 2015) may be used for extraction and enrichment of low abundance proteins in further study.

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