obtained using image scanner III. The image was processed by Quantity one (version 4.6.2, Bio-Rad, USA).

Two-dimensional electrophoresis

The purified protein pellets were resuspended in 150 μL lysis buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 40 mmol/L DTT, 0.25% [v/v] IPG buffer pH 3–10 and 0.37% [v/v] IPG buffer pH 4–7). The IPG strips were rehydrated for 24 h in rehydration buffer (7 M urea, 2 M thiourea, 2% [w/v] CHAPS, 20 mmol/L DTT, 0.3% [v/v] IPG buffer pH 4–7, 0.2% [v/v] IPG buffer pH 3–10 and 0.05% [w/v] bromophenol blue] before IEF. Followed by this procedure, it was treated on the Ettan IPGphor apparatus under the following conditions: (i) 50 V, gradient, 0.5 h; (ii) 50 V, step and hold, 2 h; (iii) 250 V, gradient, 1.5 h; (iv) 500 V, gradient, 1 h; (v) 1000 V, gradient, 2 h; (vi) 3000 V, gradient, 1.5 h; (vii) 8000 V, gradient, 3 h; (viii) 8000 V, gradient to 30000 V·h. After IEF, the IPG strips were respectively equilibrated with 10 mL of the equilibration buffer I (75 mM Tris-HCl, pH 8.8, 6 M urea, 29.3% [v/v] glycerol, 2% [w/v] SDS, 1% [w/v] DTT, 0.05% [w/v] bromophenol blue) for 15 min and then kept for another 15 min in alkylating equilibration buffer containing 2.5% (w/v) iodoacetamide instead of 1% DTT. The sealing liquid of agarose was loaded on the 12% polyacrylamide gel (C = 3%, gel dimensions 14 cm × 16 cm), and the strips were quickly transferred to the sealing liquid. As soon as the agarose sealing liquid freezing (about 15 min at 4°C), SDS-PAGE was performed under 12% polyacrylamide gel at a constant 60 V for about 1 h and 120 V for 10 h. After electrophoresis, each gel was visualized with silver staining. After the gel scanned, the images were processed by the PDQuest software (Bio-rad, UAS). The quantitative comparison of the spots was carried out by the scanner-generated spot volume and was expressed as a numeric value of optic density after subtraction of background. Student's *t*-test was also performed to compare the different groups ($P < 0.05$). Then, hierarchical cluster analysis was performed based on the expression valves of matched protein spots. The analysis was performed with SPSS version 19.0 software, a method named as Ward' method was applied, and Block distance was selected as measurement.

RESULTS AND DISCUSSION

Protein preparation

Protein sample preparation is the key factor in 2-DE analysis, the extraction method affects the electrophoretic quality such as resolution and reproducibility.[27] In the present study, due to the complexity of the natural CS protein profile and the diversity of chemical components in CS, Tris-HCl and triton X-100 instead of trichloroacetic acid/acetone were used for extraction to improve the extraction efficiency of membrane and hydrophobic proteins. Meanwhile, to remove most of polysaccharides, phenolic substances, and other secondary metabolites, the crude protein was precipitated by acetone before purified by 2D clean-up kit. As shown in Figures 1 and 2, the method of protein sample preparation was suitable for gel electrophoresis analysis. In addition, the concentrations of protein in 26 CS samples were measured according to the method of Bradford [Table 2], the data indicated that the crude protein concentrations in 26 producing areas had not obvious difference.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE has frequently been used for determination of a given protein band and the molecular mass of proteins.[28] In the present study, 26 natural CS samples were analyzed by the 12% SDS-PAGE [Figure 1]. As shown in Figure 1, the molecule weights of major proteins in CS samples were from 6.5 to 97.2 kDa. The SDS-PAGE gel images was processed by the software quantity one, the results showed that the optical density curves of proteins and the numbers of protein bands from 26 samples were a bit different [Table 2]. In reality, there existed a certain degree of difference between different samples, for example, three Tibet sample (CS6, CS7, and CS8) only owned 19, 19 and 20 protein bands, respectively, while seven Qinghai province samples had 23–27 protein bands. Furthermore, the expression values of some protein bands differ among samples, i.e., the protein bands of 6.5 kDa in CS21 and 17.0 kDa in CS23 showed high abundance, respectively. The difference of protein bands in number and abundance in CS samples might be due to the difference of growing environment, processing and storage conditions. Although there were some differences exist in the results of SDS-PAGE, it cannot identify the correlation between the diversity of soluble proteins and producing areas. Therefore, 2-DE with higher resolution of proteins separation was used for further analysis.

Two-dimensional electrophoresis

To further investigate the protein profiles of 26 batches natural CS samples, the 2-DE maps was obtained by the first IEF (IPG strip 13 cm,

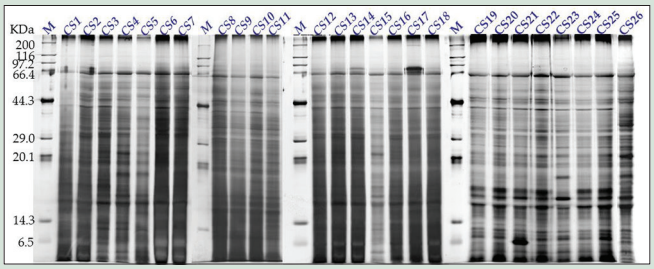


Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of natural *Cordyceps sinensis* obtained from 26 producing areas. M, molecular weights (kDa) of standard marker; CS1 to CS26, *Cordyceps sinensis* samples from different producing areas

Table 2: The concentrations of soluble proteins and numbers of sodium sulfate-polyacrylamide gel electrophoresis protein bands of 26 *Cordyceps sinensis* samples

Sample number	Protein concentration (mg/mL±SD)	Numbers of protein bands
CS1	1.27±0.02	19
CS2	1.71±0.05	19
CS3	1.34±0.07	27
CS4	1.61±0.05	23
CS5	1.17±0.07	26
CS6	2.21±0.15	19
CS7	1.98±0.03	19
CS8	1.64±0.04	20
CS9	1.58±0.03	23
CS10	1.72±0.03	22
CS11	2.35±0.04	20
CS12	2.31±0.06	23
CS13	2.21±0.09	23
CS14	2.54±0.02	25
CS15	1.45±0.11	26
CS16	2.38±0.06	24
CS17	2.16±0.05	19
CS18	1.90±0.14	20
CS19	1.18±0.01	24
CS20	1.01±0.03	25
CS21	0.96±0.02	25
CS22	1.28±0.03	24
CS23	0.95±0.11	27
CS24	0.94±0.01	23
CS25	1.04±0.08	27
CS26	0.98±0.05	26

CS: *Cordyceps sinensis*; SD: Standard deviation

pH 4–7) and the second 12% SDS-PAGE. Three typical gel images from three provinces were shown in Figure 3. By the software PDQuest, 500–1100 protein spots [Table 3] were detected in 2-DE profile maps, the pI values of main proteins in 26 batches were from 4.5 to 6.5 and the molecule weights of the major proteins were ranged from 6.5 to 100 kDa. Obviously, the numbers of protein spots were different in 26 samples, such as CS1 (Changdu of Tibet) had 939 protein spots, CS4 (Kangding of Sichuan province) owned 715, while CS23 (Guoluo of Qinghai) owned 1027 protein spots. Moreover, the abundance of protein spots from 26 batches varied greatly among different producing areas.

Table 3: Number of protein spots and matching rates of two-dimensional electrophoresis protein profiles of 26 *Cordyceps sinensis* samples

Matching mode	Number of protein spots	Number of matched spots	Matching rate/ percentage
CS1/CS5	923	430	59
CS2/CS5	732	359	50
CS3/CS5	804	293	40
CS4/CS5	715	291	40
CS5/CS5	717	717	100
CS6/CS5	599	368	51
CS7/CS5	819	415	57
CS8/CS5	550	259	36
CS9/CS5	466	237	33
CS10/CS5	536	207	28
CS11/CS5	1010	397	55
CS12/CS5	494	241	33
CS13/CS5	377	219	58
CS14/CS5	701	332	46
CS15/CS5	717	282	39
CS16/CS5	922	316	44
CS17/CS5	872	210	29
CS18/CS5	769	340	47
CS19/CS5	959	455	63
CS20/CS5	896	407	56
CS21/CS5	999	423	58
CS22/CS5	921	462	64
CS23/CS5	1027	455	63
CS24/CS5	1075	447	62
CS25/CS5	857	396	55
CS26/CS5	1011	295	41

CS: *Cordyceps sinensis*

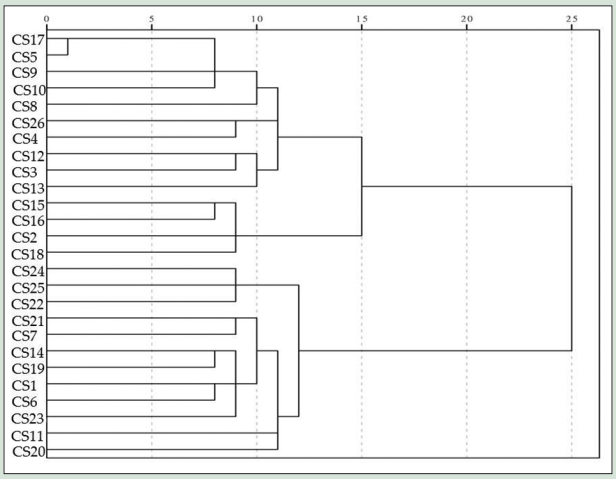


Figure 2: Dendrogram of hierarchical cluster analysis based on the matched protein spots' expression values. The hierarchical cluster was done by SPSS software. A method named as ward' method was applied, and block distance was selected as measurement

Compared with the previous report, 500–1300 acidic protein spots were characterized in the present study while only 192–298 spots in Ren'

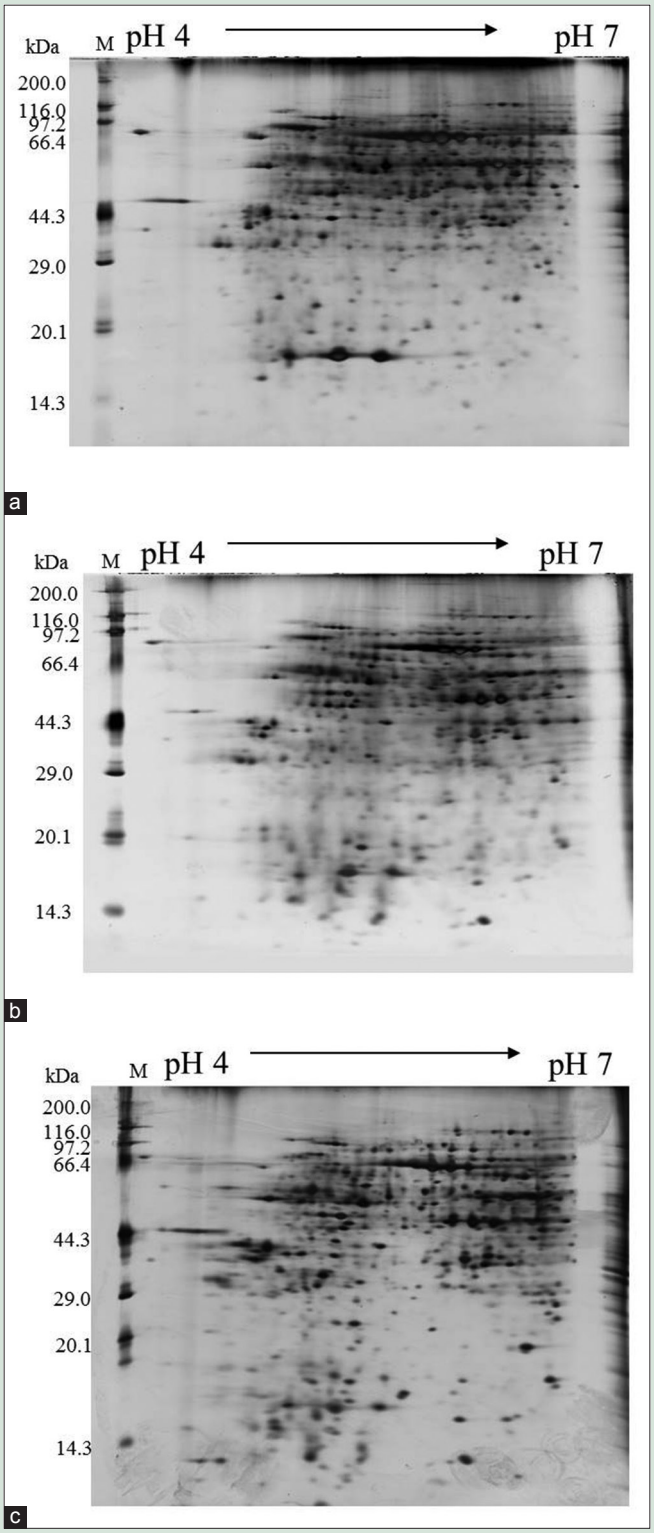


Figure 3: Typical two-dimensional electrophoresis protein profiles of natural *Cordyceps sinensis* samples from Sichuan (a, CS5), Tibet (b, CS14) and Qinghai (c, CS22) provinces. Two-dimensional electrophoresis was performed with the first IEF (pH 4–7, 13 cm) and the second 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M, molecular weights (kDa) of standard marker

study.[23] To investigate the relationship between diversity of soluble protein and producing areas, the protein spots of 26 samples was matched with the protein spots of CS5 (Dao Cheng, Sichuan province), the matching rate was 28%–63% [Table 3]. The low matching rate of different samples may be attributed to the collecting time, habitat or processing conditions. Furthermore, based on the matched protein spots' expression valves, 26 batches of CS samples were separated into two categories by hierarchical cluster analysis [Figure 2]. As shown in Figure 2, 26 batches of natural CS samples were mainly distributed into Tibet and Qinghai two categories, while Sichuan samples were scattered into these two categories. Particularly, CS1 and CS14 were significantly different from other Tibet samples, may be result of abiotic stress[29] and natural variability.[30] Therefore, the results of hierarchical cluster analysis showed that 26 producing areas of natural CS had a certain relationship with the diversity of soluble proteins.

CONCLUSIONS

In the present study, the diversity of soluble proteins in natural CS from 26 different areas of China was characterized using SDS-PAGE and 2-DE analysis. The results indicated that the protein bands in SDS-PAGE were a little different in 26 samples. Furthermore, the results of hierarchical cluster analysis based on the matched protein spots on 2-DE profiles showed that the common characters of matched protein spots had a relationship with producing areas. Further research should be done to identify the active and characteristic proteins of each producing area to increase the understanding of protein components in natural CS.

Financial support and sponsorship

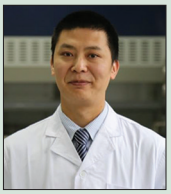
This work was supported by the National Natural Science Foundation of China (21275169 and 81202886), the Natural Science Foundation Project of CQ CSTC (cstc2015jcyjA10044), project no. CQDXWL-2014-Z007 supported by the Fundamental Research Funds for the Central Universities.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Wu R, Gao JP, Wang HL, Gao Y, Wu Q, Cui XH. Effects of fermented *Cordyceps sinensis* on oxidative stress in doxorubicin treated rats. Pharmacogn Mag 2015;11:724-31.
2. Jiraungkoorskul K, Jiraungkoorskul W. Review of naturopathy of medical mushroom, *Ophiocordyceps sinensis*, in sexual dysfunction. Pharmacogn Rev 2016;10:1-5.
3. Liu Y, Wang J, Wang W, Zhang H, Zhang X, Han C. The chemical constituents and pharmacological actions of *Cordyceps sinensis*. Evid Based Complement Alternat Med 2015;2015:575063.
4. Li Y, Wang XL, Jiao L, Jiang Y, Li H, Jiang SP, *et al*. A survey of the geographic distribution of *Ophiocordyceps sinensis*. J Microbiol 2011;49:913-9.
5. Yue K, Ye M, Zhou Z, Sun W, Lin X. The genus *Cordyceps*: A chemical and pharmacological review. J Pharm Pharmacol 2013;65:474-93.
6. Tuli HS, Sharma AK, Sandhu SS, Kashyap D. Cordycepin: A bioactive metabolite with therapeutic potential. Life Sci 2013;93:863-9.
7. Hu H, Xiao L, Zheng B, Wei X, Ellis A, Liu YM, *et al*. Identification of chemical markers in *Cordyceps sinensis* by HPLC-MS/MS. Anal Bioanal Chem 2015;407:8059-66.
8. Fan H, Yang FQ, Li SP. Determination of purine and pyrimidine bases in natural and cultured *Cordyceps* using optimum acid hydrolysis followed by high performance liquid chromatography. J Pharm Biomed Anal 2007;45:141-4.
9. Yang FQ, Guan J, Li SP. Fast simultaneous determination of 14 nucleosides and nucleobases in cultured *Cordyceps* using ultra-performance liquid chromatography. Talanta 2007;73:269-73.
10. Yang FQ, Li SP. Effects of sample preparation methods on the quantification of nucleosides in natural and cultured *Cordyceps*. J Pharm Biomed Anal 2008;48:231-5.
11. Yang FQ, Ge L, Yong JW, Tan SN, Li SP. Determination of nucleosides and nucleobases in different species of *Cordyceps* by capillary electrophoresis-mass spectrometry. J Pharm Biomed Anal 2009;50:307-14.
12. Ghatnur SM, Parvatam G, Balaraman M. Culture conditions for production of biomass, adenosine, and cordycepin from *Cordyceps sinensis* CS1197: Optimization by desirability function method. Pharmacogn Mag 2015;11 Suppl 3:S448-56.
13. Zuo HL, Chen SJ, Zhang DL, Zhao J, Yang FQ, Xia ZN. Quality evaluation of natural *Cordyceps sinensis* from different collecting places in China by the contents of nucleosides and heavy metals. Anal Methods 2013;5:5450-6.
14. Ye M, Hu Z, Fan Y, He L, Xia F, Zou G. Purification and characterization of an acid deoxyribonuclease from the cultured mycelia of *Cordyceps sinensis*. J Biochem Mol Biol 2004;37:466-73.
15. Chen X, Ding ZY, Wang WQ, Siu KC, Wu JY. An antioxidative galactomannan-protein complex isolated from fermentation broth of a medicinal fungus Cs-HK1. Carbohydr Polym 2014;112:469-74.
16. Wu JY, Chen X, Siu KC. Isolation and structure characterization of an antioxidative glycopeptide from mycelial culture broth of a medicinal fungus. Int J Mol Sci 2014;15:17318-32.
17. Wang J, Liu YM, Cao W, Yao KW, Liu ZQ, Guo JY. Anti-inflammation and antioxidant effect of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*, in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Metab Brain Dis 2012;27:159-65.
18. Qi W, Zhang Y, Yan YB, Lei W, Wu ZX, Liu N, *et al*. The protective effect of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis*, on diabetic osteopenia in alloxan-induced diabetic rats. Evid Based Complement Alternat Med 2013;2013:985636.
19. Yu SH, Wang XH, Li SP, Ji H. The comparison on soluble protein of *Cordyceps sinensis* (Berk.) Sacc. from different localities and its fermentative hyphae from different factories. J Plant Resour Environ 2000;9:59-61.
20. Shi JH, Dang HN, Wan Y, Bao CJ. Comparative analysis of protein from nature *Cordyceps sinensis* and cultured *Cordyceps* mycelia. Pharm Biotechnol 2003;10:304-7.
21. Ren Y, Qiu Y, Wan DG, Lu XM, Guo JL. Preliminary study on correlation between diversity of soluble proteins and producing area of *Cordyceps sinensis*. Zhongguo Zhong Yao Za Zhi 2013;38:1375-7.
22. Hu YH, Li X, Shao WL. Renaturation and active staining of xylanase from *Volvariella volvacea* after SDS-PAGE. J Wuxi Univ Light Ind 2003;22:102-3.
23. Ren Y. Reasearch on the Species of Valuble Chinese Medicine "*Cordyceps sinensis*" and Its Protein Components. Ph.D. Thesis. Chengdu University of TCM, Chengdu, China; 2013.
24. Nehete JY, Bhambar RS, Narkhede MR, Gawali SR. Natural proteins: Sources, isolation, characterization and applications. Pharmacogn Rev 2013;7:107-16.
25. Satish A, Sairam S, Ahmed F, Urooj A. *Moringa oleifera* Lam.: Protease activity against blood coagulation cascade. Pharmacognosy Res 2012;4:44-9.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
27. Blackstock WP, Weir MP. Proteomics: Quantitative and physical mapping of cellular proteins. Trends Biotechnol 1999;17:121-7.
28. Carter J, Petersen BP, Printz SA, Sorey TL, Kroll TT. Quantitative application for SDS-PAGE in a biochemistry lab. J Chem Educ 2013;90:1255-6.
29. Abreu IA, Farinha AP, Negrão S, Gonçalves N, Fonseca C, Rodrigues M, *et al*. Coping with abiotic stress: Proteome changes for crop improvement. J Proteomics 2013;93:145-68.
30. Natarajan SS. Natural variability in abundance of prevalent soybean proteins. Regul Toxicol Pharmacol 2010;58 3 Suppl:S26-9.



Feng-Qing Yang

ABOUT AUTHOR

Dr. Feng-Qing Yang graduated from the China Pharmaceutical University, Nanjing, China, and he obtained the M.D. and Ph.D. degrees from the University of Macau, Macau. He has been a Professor of the School of Chemistry and Chemical Engineering at the Chongqing University, Chongqing, China, since 2014. He has published more than 70 SCI papers (h-index 23). His research interests include analytical chemistry (theory and application in pharmaceutical analysis), modern chromatographic separation science (HPLC-DAD/ELSD, GC-MS, LC-MS, etc.), modern sample preparation techniques (SFE, PLE, etc.), spectrometry (UV, MS, etc.), analytical device modifications, natural products, and quality control of traditional Chinese medicines.