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ORIGINAL ARTICLE

Analysis of Soluble Proteins in Natural *Cordyceps sinensis* from Different Producing Areas by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Two-dimensional Electrophoresis

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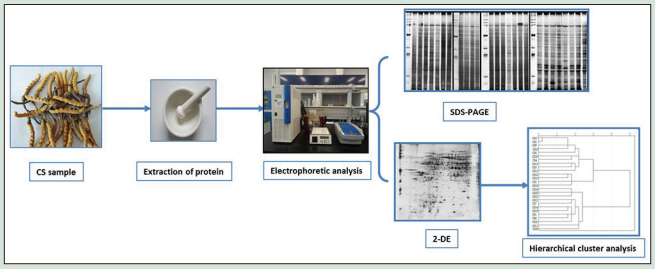
ABSTRACT

Background: As one of the bioactive components in *Cordyceps sinensis* (CS), proteins were rarely used as index components to study the correlation between the protein components and producing areas of natural CS. **Objective:** Protein components of 26 natural CS samples produced in Qinghai, Tibet, and Sichuan provinces were analyzed and compared to investigate the relationship among 26 different producing areas. **Materials and Methods:** Proteins from 26 different producing areas were extracted by Tris-HCl buffer with Triton X-100, and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis (2-DE). **Results:** The SDS-PAGE results indicated that the number of protein bands and optical density curves of proteins in 26 CS samples was a bit different. However, the 2-DE results showed that the numbers and abundance of protein spots in protein profiles of 26 samples were obviously different and showed certain association with producing areas. **Conclusions:** Based on the expression values of matched protein spots, 26 batches of CS samples can be divided into two main categories (Tibet and Qinghai) by hierarchical cluster analysis.

Key words: Hierarchical cluster analysis, natural *Cordyceps sinensis,* sodium dodecyl sulfate-polyacrylamide gel electrophoresis, soluble protein, two-dimensional electrophoresis

SUMMARY

- The number of protein bands and optical density curves of proteins in 26 *Cordyceps sinensis* samples were a bit different on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles
- Numbers and abundance of protein spots in protein profiles of 26 samples were obvious different on two-dimensional electrophoresis maps
- Twenty-six different producing areas of natural *Cordyceps sinensis* samples were divided into two main categories (Tibet and Qinghai) by Hierarchical cluster analysis based on the values of matched protein spots.



Abbreviations Used: SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 2-DE: Two-dimensional electrophoresis, *Cordyceps sinensis*: CS, TCMs: Traditional Chinese medicines

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INTRODUCTION

Cordyceps sinensis (CS) (Dongchongxiacao), a caterpillar entomopathogenic fungus-host larva complex, is recognized as one the most famous tonic traditional Chinese medicines for centuries. Natural CS, which has various pharmacological effects including anticonvulsant activity, anti-inflammatory, antitumor, antioxidant, hypoglycemic, and improving sexual functions,[1-3] is mainly produced in Tibet, Qinghai, Sichuan, and Yunnan provinces of China.[4] Usually, based on the size and color of the fruiting body and stromata, or even based on their area of production (e.g., products of the province of Tibet are thought to be the best), the price of natural CS is varied significantly. So comparison on the quality variation between natural CS samples from different producing areas will be of interest to the consumers. Up to date, several chemical compounds such as nucleosides, sterols and polysaccharides were often used as the markers for quality control of CS.[5-7] Especially, the nucleosides and their various analytical methods have been used for the quality control of natural CS.[8-12] In reality, the contents of nucleoside among different producing areas were similar. For example, Zuo *et al.* developed a high-performance liquid chromatography-diode array detection method for determination of the contents of six nucleosides in natural CS from 17 producing areas, the results showed that it is difficult to distinguish different CS samples using nucleosides as chemical markers.[13]

On the other hand, proteins are thought to be one of the major active components in *Cordyceps*, which possess various pharmacological activities, such as acid DNase activity,[14] anti-oxidation,[15,16] anti-inflammation,[17] and anti-obesity.[18] Meanwhile, previous study

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reported that the proteins were significantly different in natural and cultured CS samples analyzed by cellulose acetate film electrophoresis and polyacrylamide vertical slab gel electrophoresis.[19,20] Furthermore, Ren *et al*.[21] reported on the diversity of soluble proteins of natural CS from three producing areas by two-dimensional electrophoresis (2-DE), and there may be a certain correlation between producing areas and the diversity of soluble proteins, but the sample numbers were limited. In addition, compared with the high performance gel filtration chromatography, capillary zone electrophoresis and capillary isoelectric focusing (IEF), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and 2-DE as the classical methods with high resolution and good reproducibility, are usually used for analysis of proteins from medicinal fungi[22,23] and plant materials.[24,25] Therefore, in this work, proteins from natural CS produced in 26 producing areas were characterized and compared based on SDS-PAGE and 2-DE analysis, and the correlation between the diversity of soluble proteins and 26 producing areas was studied by hierarchical cluster analysis based on the expression values of matched protein spots in 2-DE protein profiles.

MATERIALS AND METHODS

Regents and chemicals

2-DE cells used for IPG strips (pH 4–7, 13 cm), drystrip cover fluid, 2D clean-up kit and IPG buffer (pH 3–10 and pH 4–7) were obtained from GE Healthcare (GE Healthcare Bio-Science, Uppsala, Sweden). N, N-methylene-bis acrylamide, N, N-methylene-bis acrylamide, 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), acrylamide, dithiothreitol (DTT), iodoacetamide, low melting-temperature agarose, 2-aimino-2-(Hydroxymethyl)-1,3-propanediol (Tris), glycine, urea, thiourea, coomassie brilliant blue G-250, SDS and N,N,N',N'-Tetramethylethylenediamine were purchased from Sangon Biotech Co., Ltd., (Shanghai, China). Protein marker was obtained from Microgram-Tiantai (Chengdu, China). The ampholytes (pH 3-10 and pH 4-7) 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, ammonium persulfate (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). The protease inhibitor cocktail (P-9599) was from Sigma. The ultrapure water used for experiment was produced by Milli-Q water purification system (DZG-303A, Ai-ke, China).

Instruments

Electrophoresis power supple-EPS 601, vertical electrophoresis system SE 600 Ruby (14 cm × 16 cm gel dimension), Multiphor II, Image Scanner III, Ettan IPGphor 3 systems and manifold were purchased from GE Healthcare (GE Healthcare Bio-Science, Uppsala, Sweden). High-speed refrigerated centrifuge was 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 shaking table for electrophoresis gel immobilization, sensitization, staining, and decolorization was from Shanghai Yarong Biochemical Works (Shanghai, China).

Protein sample preparation

Twenty-six mature natural CS samples collected from different production places are listed in Table 1. The identification of CS fruiting bodies was certified by the corresponding author and was deposited at the Department of Pharmaceutics, School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

Table 1: The production places of natural *Cordyceps sinensis*

Sample number	Collection places
CS1	Chang Du, Tibet province
CS2	Ka Ma Duo of Lei Wu Qi, Tibet province
CS3	Ding Qing, Tibet province
CS4	Kang Ding, Sichuan province
CS5	Dao Cheng, Sichuan province
CS6	Ta Zi Ba of Li Tang, Sichuan
CS7	A Jia Gou of Li Tang, Sichuan
CS8	Sha De, Sichuan province (normal *Cordyceps*)
CS9	Sha De, Sichuan province (black *Cordyceps*)
CS10	Ya Jia Geng of Kang Ding, Sichuan province
CS11	Li Tang, Sichuan province
CS12	Bu Ta of Dingqing, Tibet province
CS13	Xue Ta of Chang Mao Ling of Lei Wu Qi, Tibet province
CS14	Ka Beng of Gan Yan of Ding Qing, Tibet province
CS15	Na Long of Ka Ma Duo of Lei Wu Qi, Tibet province
CS16	Duo Su of Gang Se of Lei Wu Qi, Tibet province
CS17	Ga Ji of Lei Wu Qi, Tibet province
CS18	La En of Gang Se of Lei Wu Qi, Tibet province
CS19	Yu Shu, Qinghai province
CS20	Zha Duo of Yu Shu, Qinghai province
CS21	Yu Shu, Qinghai province
CS22	Ma Mi of Guo Luo, Qinghai province
CS23	Jing Rang of Guo Luo, Qinghai province
CS24	Xue Shan of Guo Luo, Qinghai province
CS25	Qu Ma Lai of Yu Shu, Qinghai province
CS26	Kang Ding, Sichuan province

CS: *Cordyceps sinensis*

The sample powder (0.2 g) was grounded into fine homogeneous powder in liquid nitrogen and extracted with 6 mL of Tris-HCl buffer (60 mmol/L Tris was adjusted to pH 8.3 by HCl) including 60 mmol/L Tris-HCl, pH 8.3, 20 mmol/L DTT, 0.5% (v/v) Triton X-100 and 0.1% (v/v) protease inhibition in the mortar grinding for 30 min. The homogenate was transferred to microtube and centrifuged at $1.7*10^4$ ×*g* for 15 min at 4°C. The supernatant was collected and purified by 2D clean-up kit (including precipitant, co-precipitant, wash buffer, and wash additive) after acetone precipitant. The contents of extracted protein solutions and purified protein solutions were measured by the method of Bradford.[26] Transfer 100 μL above crude protein supernatant 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 at $1.7*10^4$ ×*g* for 5 min at 4°C, the protein pellets were dispersed in 25 μL ultra-pure water, and then add 1 mL pro-chilled wash buffer and 5 μL wash additive to the suspension. The suspension should be incubated at −20°C for at least 30 min. After centrifuging and carefully discarding the supernatant, the pellet was resuspended in SDS-buffer (80 mmol/L Tris-HCl, pH 6.8, 2% [w/v] SDS, 10 mmol/L DTT, 20% [v/v] glycerol and 0.05% [w/v] bromophenol blue) or protein lysate for electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ten microliters ultra-pure water and 10 μL SDS loading buffer were added into above purified pellet protein samples and heated at 100°C for 10 min, then centrifuged at $1.2*10^4$ ×*g* for 1 min (4°C). The denatured protein solution was loaded in each lane and run on 12% acrylamide gel (gel dimensions 14 cm × 16 cm, crosslinker concentration C = 3%). Standard protein marker was applied to each gel. Gels were fixed overnight and stained with silver-staining. Digital image of gels was

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,

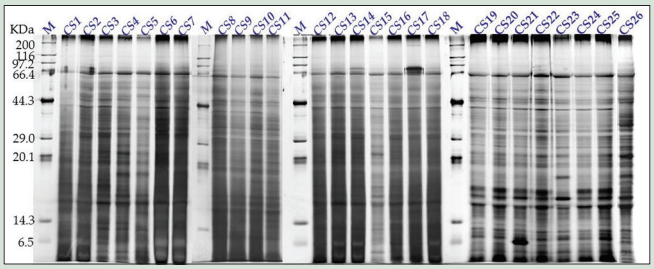

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles of natural *Cordyceps sinensis* obtained from 26 producing areas. M, molecular weights (kDa) of standard marker; CS1 to CS26, *Cordyceps sinensis* samples from different producing areas

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*

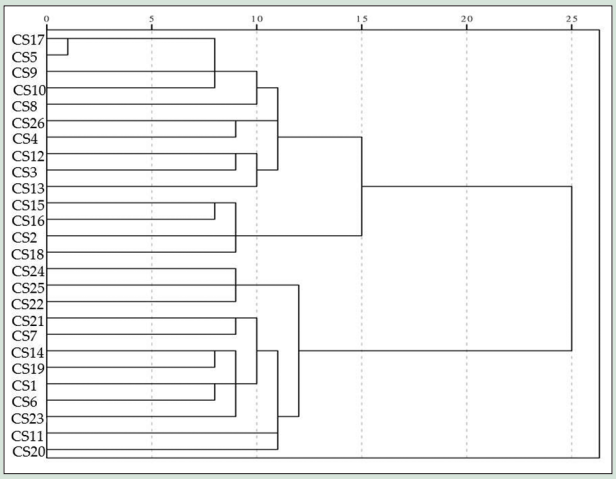

Figure 2: Dendrogram of hierarchical cluster analysis based on the matched protein spots' expression values. The hierarchical cluster was done by SPSS software. A method named as ward' method was applied, and block distance was selected as measurement

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

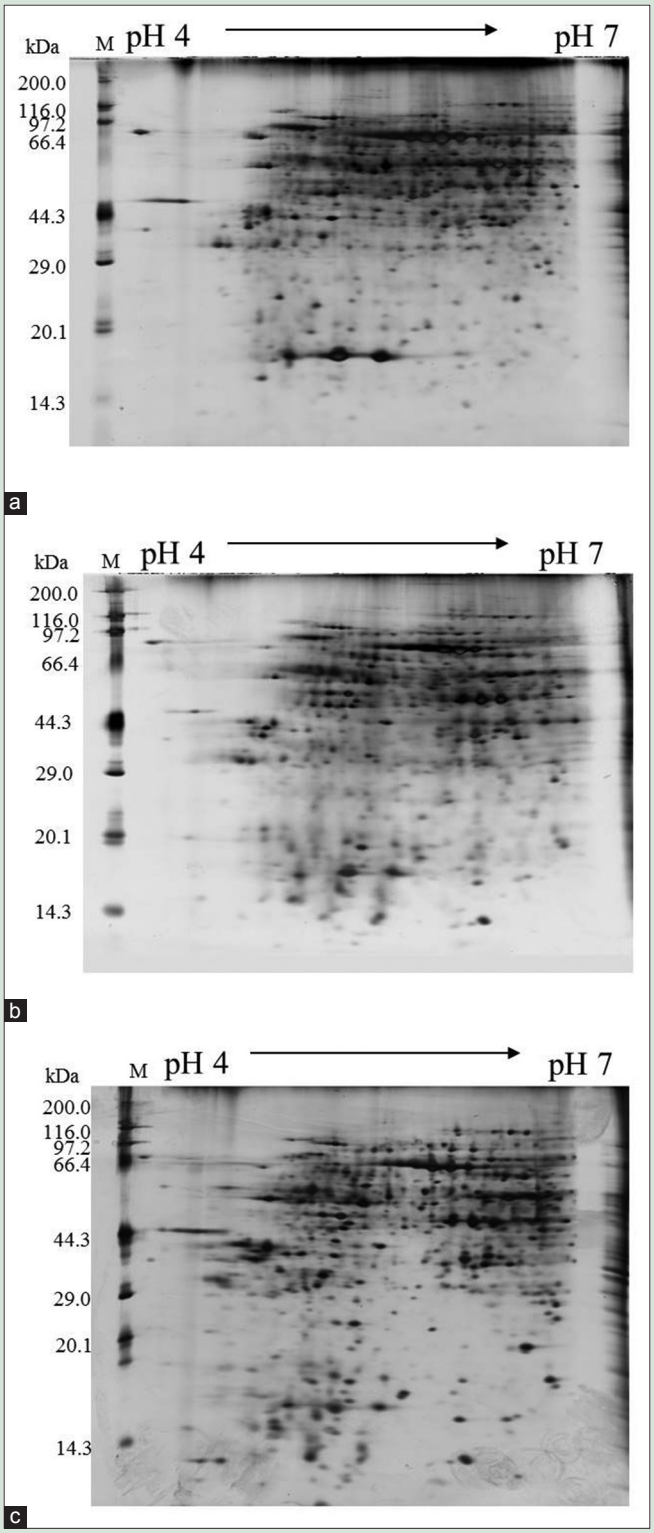

Figure 3: Typical two-dimensional electrophoresis protein profiles of natural *Cordyceps sinensis* samples from Sichuan (a, CS5), Tibet (b, CS14) and Qinghai (c, CS22) provinces. Two-dimensional electrophoresis was performed with the first IEF (pH 4–7, 13 cm) and the second 10% sodium dodecyl sulfate-polyacrylamide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

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Conflicts of interest

There are no conflicts of interest.

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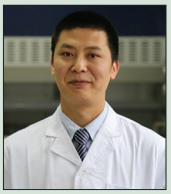

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