Proteomic identification of marker proteins and its application to authenticate *Ophiocordyceps sinensis*

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Received: 12 February 2018 / Accepted: 25 April 2018 / Published online: 8 May 2018
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Abstract

Ophiocordyceps sinensis (*O. sinensis*) is a highly valuable fungus because of its nutritious and medicinal properties. The objective of this study was to identify protein markers using a proteomics approach followed by the development of an immunoassay to authenticate *O. sinensis*. Four authentic *O. sinensis* samples collected from four production regions and four counterfeit samples were examined individually. Overall 22 characteristic proteins of *O. sinensis* were identified by two-dimensional electrophoresis (2-DE) coupled with the matrix-assisted laser desorption/ionization-time-of-light mass spectrometry (MALDI-TOF/MS). Three authentic *O. sinensis* samples and three counterfeit samples were examined by the couple of alkaline native gradient PAGE (AN-PAGE) and electrospray ionization quadrupole-time-of-light mass spectrometry (ESI-Q-TOF/MS). One distinctive protein was identified to be cyanate hydratase, which was also one of the 22 distinctively characteristic proteins of *O. sinensis* and termed as IP4 in 2-D gel. Due to the abundance and high specificity of IP4, it was isolated and purified. Its purity was evaluated by high performance liquid chromatography (HPLC) and identified by ESI-Q-TOF/MS. Then the purified IP4 was used to produce polyclonal antibodies in BALB/c mice. The specificity of the anti-IP4 antibody was evaluated by an association of double immunodiffusion (DID) and indirect ELISA assay. Then an indirect enzyme linked immunosorbent assay (ELISA) was preliminarily developed to authenticate *O. sinensis* by detecting IP4. To evaluate the feasibility and accuracy of this method, three authentic *O. sinensis* samples and three counterfeits were analyzed. The P/N ratios (dividing the sample OD_{450nm} by the OD_{450nm} of negative controls) of three authentic *O. sinensis* samples were above 8, while, those of three counterfeits were lower than 1. These results indicated that the established ELISA assay based on proteomic protocols detection of protein markers might have a great potential in the authentication and also quality assessment of *O. sinensis* in those commercial products.

Keywords *O. sinensis* · Authenticate · Proteomic protocol · Indirect ELISA

Introduction

Ophiocordyceps sinensis (Berk.) Sacc. (*O. sinensis*), belonging to ascomycetes family, is one of the most commercially valued fungus. It is native to the alpine habitats (3600–5000 m in elevation) on the Tibetan plateau, India, Bhutan and Nepal (Zhang et al. 2012). *O. sinensis*

specifically colonizes ghost moth caterpillars (*Thitarodes* spp.), making a parasitic complex that comprises the remains of the insect larva and fungal sexual stroma (En-Hua Xia et al. 2017). This fungus has been used as a traditional tonic food supplementation and traditional Chinese medicine (TCM) in treatment of bronchial and lung inflammation, sexual impotence diseases and renal dysfunction, etc. for over 2000 years in Asian countries (En-Hua Xia et al. 2017; Xu et al. 2016; Hardeep et al. 2014; Qian et al. 2012). This fungus has attracted increasing attentions due to its various bioactivities, including as immunomodulation, antitumor, antioxidant, cardiovascular protection, and so forth (Xu et al. 2016).

Due to the popularity of *O. sinensis*, its natural populations have been over-harvested. Moreover, it is slow growing, requiring 3 to 4 years to mature in wild. As a result, the

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current production of *O. sinensis* cannot satisfy the market demand and the unite price of high-quality *O. sinensis* is as high as USD 53 700 per kg in China (Dong et al. 2015; Jinlin Guo et al. 2012). Consequently, the related species of *O. sinensis*, such as *Cordyceps militaries*, *Cordyceps gunnii*, *Cordyceps liangshanensis* are frequently used as substitutes or adulterants (Jinlin Guo et al. 2012, 2015). However, conventional morphological based method are unable to distinguish between *O. sinensis* and its adulterant counterparts due to their similar appearance (Heinrich and Anagnostou 2017; James et al. 2016; Huijuan Liu, Haobin; Hu et al. 2011). Recently, many methods were developed for the analysis of various components, most of them are based on the liquid chromatography (LC), capillary electrophoresis (CE), near infrared spectroscopy (NIR), and liquid chromatography mass spectrometry (LCMS) techniques, which has been widely used to identify medicinal herbs (Wang et al. 2013; Zhang et al. 2012a, b; Yang et al. 2010; Qian and Li 2017). Current methods used for the identification of medicinal herbs are mainly relied on DNA sequencing, but these methods are unsuitable for processed products where the DNA is lack or degraded (James et al. 2016; Xiang et al. 2013; Zhang et al. 2013; Jin et al. 2013).

Recently, some researches focused on alternative components that attribute to the nutritious and medical benefits. Hankun Hu et al. employed HPLC-MS/MS and pinpointed six unique chemical markers of *O. sinensis* for quantification and quality assessment (Hankun et al. 2015). Besides, Shiwei Zhang et al. used differential proteomic method to identify protein markers of *O. sinensis*, which could be used for future immunoassay development (Zhang et al. 2016). For a thorough investigation of the characteristic protein markers of *O. sinensis*, we examined four authentic samples and four counterfeit samples with 2-DE/MS (Bhardwaj and Yadav. a common protein extraction protocol for proteomic analysis: 2013; Zhang et al. 2010), which resulted in the discovery of 22 proteins that were unique to *O. sinensis*. Outcomes from this analysis facilitated our preliminary effort in developing an indirect ELISA for *O. sinensis* authentication.

Materials and apparatus

Four *O. sinensis* samples were collected from their native habitats, respectively, freeze dried for 2 days, and stored at -20°C (Table 1). Four counterfeit samples were provided by Prof. Guo (Table 1). All samples were collected when mature. Male BALB/c mice with the age of 8 to 10 weeks were housed under specific pathogen-free conditions in accordance with the guideline of the Experimental Animal Center (Sichuan University, Chengdu, China.). Ready-prep™ 2-D Cleanup Kit was purchased from Bio-Rad (Hercules, California, USA). Protein marker and other chemicals

Table 1 Information of *O. sinensis* and substitutes samples

	Code	Name	Habitat
1	OS1	<i>O. sinensis</i>	Yajiangeng, Sichuan province, China
2	OS2	<i>O. sinensis</i>	Linzhi, Tibet, China
3	OS3	<i>O. sinensis</i>	Qinghai, China
4	OS4	<i>O. sinensis</i>	Maerkang, Sichuan province, China
6	CA	<i>C. agriota</i>	Liangshan, Sichuan province, China
7	CL	<i>C. liangshanensis</i>	Liangshan, Sichuan province, China
8	CG	<i>C. gunnii</i>	Lijiang, Yunnan province, China
9	CM	<i>C. militaris</i>	Fujian, China

for electrophoresis were purchased from Thermo Fisher (Waltham, Massachusetts, USA). A PowerPac HV system (Bio-Rad) was used for the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); Protean IEF Cell and two-dimensional electrophoresis (2-DE) system were purchased from Bio-Rad (Hercules, California, USA). Micro TOF-QII™ ESI-Q-TOF LC/MS was purchased from Bruker Daltonics (Germany), which was used for mass spectrometry (MS) analysis.

Methods

Crude protein extraction

Crude protein extraction was performed according to John E Coligan et al (John and Coligan 2007; Ren yan 2013). Briefly, 0.5 g of ground samples (the mixture of caterpillar body and stroma) were dissolved in 5 mL pre-cold protein extract solution (phosphate buffer saline (PBS) 0.02 mol/L, pH 7.2, 0.2% Tween 80, 0.2% β -mercaptoethanol, 1 mM PMSF), and centrifuged (10,000 rpm, 15 min) at 4°C . Supernatant and sediment were collected, and the sediment was treated with a ten-fold volume of pre-cold 10% TCA/acetone (0.07% DTT). The mixture was subsequently vortexed for 10 s, incubated at -20°C for 1 h and centrifuged (12,000 g, 15 min) at 4°C . The supernatant was discarded and the precipitate was washed twice with pre-cold acetone solution. The precipitate was then dried at room temperature and dissolved in PBS. The protein solution was purified by Ready-prep™ 2-D Cleanup Kit (Bio-Rad, USA) prior to rehydration and stored at -80°C for use. The concentration of total protein was quantified by the BCA method by measuring the $\text{OD}_{490\text{nm}}$ in an ELISA plate reader (Thermo Fisher Scientific, USA) (Kruger 2009). Bovine serum albumin (BSA) was used as the standard.

2-DE and MALDI-TOF-TOF/MS

The protein solution was dissolved in the rehydration buffer (8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) methylammonio]-1-propanesulphonate (CHAPS), 50 mM DTT and 0.5% ampholyte). In this study, six loading quantities (i.e., 400, 500, 600, 700, 800 and 900 µg) of protein samples were compared. The first dimensional IEF was performed using 17 cm nonlinear IPG strips, and the isoelectric points (pI) of the nonlinear IPG strip was 3–10. This experiment was also carried out with a current setting of 50 µA / strip using a protean IEF cell at 20 °C. The IEF procedure was as follows, 20 °C active hydration for 12 h, 250 V for 0.5 h, 500 V for 0.5 h, 1000 V for 2 h, 10,000 V for 5 h, 10,000 V for 70,000 VH and 500 V for random time. 2-DE method was carried out according to the method of Grog et al. (John and Coligan 2007; Ren yan 2013; Kruger 2009; LIG and uan-jun 2007, 2018; Song et al. 2007; Grog et al. 2004). A 12% SDS-PAGE was used for secondary separation and the SDS-PAGE steps were as follows: 50 V for 1 h and 150 V for 9 h. SDS-PAGE gels were dyed by Coomassie Brilliant Blue (CBB) G-250, and ultrapure water was used for discoloration. Precision Plus Protein molecular weight standard (Thermo Fisher, USA) was applied to each gel. Finally, the gels were scanned on UMAX PowerLook 2100XL scanner. Image analysis was processed by PAQuest™ 8.0.1 software (Bio-Rad, USA) and statistics were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL), data was presented as $\bar{x} \pm s$.

Each differential spot was excised, digested with in-gel tryptic-digestion and identified by MALDI-TOF-TOF/MS according to Katayama et al. (2001). The resulting peptide fingerprints were used to identify proteins on the basis of their matches to proteins in insect and fungi against the NCBI nr database (<https://www.ncbi.nlm.nih.gov/>) by Mascot v2.1 engine.

Alkaline native gradient PAGE

Crude protein extraction was also performed according to John and Coligan (2007; Ren yan 2013). Crude protein was extracted from a 2 mg sample, and BCA method was used to determine protein content. 20 µg protein sample was loaded and separated by AG-PAGE (5% to 15%, 1.5M Tris-HCl, pH 8.8). After electrophoresis, lane 1, 2 and 3 were isolated and stained with CBB G-250 for 30 min. The differential band between authentic *O. sinensis* samples and counterfeits was cut off from the unstained lane 4 and 7, and dialyzed overnight with a dialysis bag against the electrophoresis buffer with a molecular weight cut-off at 8000 Da (Hunag yi 2003). Then, the isolated protein was evaluated for its purity by HPLC (Wang Hao et al. 2009) and identified by ESI-Q-TOF/MS. The recovery solution of the isolated protein was

diluted to 300 µg/ml using physiological saline, filtered with 0.22 µm Millipore membrane, and packaged in 0.5 mL /bottle and stored at 4 °C.

Production and characterization of polyclonal antibodies

Three mice were pre-injected subcutaneously with 200 µL ddH₂O emulsified with complete Freund's adjuvant at a 1:1 ratio at multi-point under limbs, respectively. After two weeks, mice received an injection of 60 µg antigen in incomplete Freund's adjuvant in a 1:1 ratio every 2 weeks. Immunization is stopped after 6 days, mice were sacrificed, and the anti-sera was collected from eyeballs (Zhu 2014). All tests were performed at the same place and the samples were processed in triplicates. Mice injected with saline only were used as negative control. The immune response was monitored by testing the titer of polyclonal antibody in mouse serum using indirect ELISA and DID (Azevedo et al. 2014). In indirect ELISA assay, a 96-well was coated with 100 µL (5 µg/ml) antigen in carbonate buffer and incubated for 5 h at 37 °C. 100 µL of a series of twofold dilution (1:4 to 1:1024) of anti-IP4 anti-sera were added and incubated at 37 °C for 1 h. For each test, the control mice sera and PBS were used in parallel as negative control and blank, respectively. The best positive dilution multiple of the anti-sera was considered to be the titer.

Establishment of an indirect ELISA

We then establish a preliminary, indirect ELISA to distinguish authentic from counterfeit *O. sinensis* samples using procedure described as follows. 100 µL of a eight serial two-fold dilutions of IP4 (starting from 2 mg/ml) with carbonate buffer 0.01 M PBS (pH 7.4), 0.05 mol/l Tris-HCl (pH 8.5), and 0.05 mol/l normal saline (pH 9.6) was incubated at 37 °C for (2, 5 h or 5 h overnight at 4 °C) in a humid chamber. Afterwards, coating solution was removed by aspiration, each wells was rinsed twice with dH₂O, and was flipped dry on paper towel. Each well was then blocked by 200 µL blocking buffer (4% PBS, 1% BSA-PBS and 1% BSA-PBST). After incubation for 25, 50 and 75 min at 37 °C, all the wells were washed three times with 0.01 M PBST (pH 7.4 containing 0.05% Tween-20) to remove the blocking solution. The wells were then rinsed twice with ddH₂O and were dried again by testing the plate upside down on paper towel. 100 µL of anti-serum dilution (1:128) was added to each well, and incubated at room temperature for 1 h. Finally, antibody solution was removed and wells were rinsed with washing buffer three times followed by adding 100 µL anti-horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (dilutions: 1:2000, 1:3000, 1:4000, 1:5000, and 1:6000) to each well for incubation at room temperature

Fig. 1 2-DE map of 600 µg total protein, separated by IEF on 7 cm pI gradient 3–10 IPG strip, 12% SDS-PAGE, stained with Coomassie Brilliant Blue (CBB). Proteins extracted from *O. sinensis* collected from four habitats and four substitutes. **a–d** correspond to sample codes in sequence: OS1, OS4, OS2 and OS3, respectively. **e–h** correspond to sample codes in sequence: CL, CA, CG and CM. **I**: differential spots between *O. sinensis* and substitutes

for 30, 45, 60 and 90 min. Then, the antibody solution was removed, and the wells were rinsed with washing buffer five times. Each plate was further washed three times by washing buffer and dried as before. OD_{450nm} was measured by Spectromax 250 microplate spectrophotometer (Molecular Devices, USA) and results were normalized by dividing the sample OD_{450nm} by the OD_{450nm} of negative control (P/N). A ratio that is greater than two was considered as positive, whereas a ratio that is less than one was regarded as negative or nonreactive.

To evaluation this method, three authentic samples of *O. sinensis* and three counterfeit samples were examined. First, the purified IP4 solution was diluted (0.1 to 0.6 mg/ml) with 0.05 mol/l normal saline (pH 9.6) to set up the standard curve. All incubation was carried out at 37 °C in triplicates. For each test, a positive control (100 µL antigen) and negative control (100 µL PBS) were included.

Results and discussion

Proteomic analysis

The conditions of 2-DE were optimized. A protein sample loading quantity of 600 µg was separated through 17 cm pI 3–10 IPG strips, and the filter paper pads were replaced beneath the electrodes to remove salts. The 2-D gels resolved 775 ± 27 , 798 ± 25 , 634 ± 21 and 712 ± 18 spots, respectively, in OS1, OS2, OS3 and OS4 samples (Table 1; Fig. 1a–d), which scattered a pI of 4.0 to 9.0 and a molecular weight of 30 to 90 kDa. The proteomic patterns of *O. sinensis* samples from four habitats were similar in spot amount, molecular size and pI value. Differences in spots distribution and concentrations of protein spots could likely caused by growing environment, which is in accordance with previous findings (Zhang et al. 2016). Nevertheless, a huge difference was found in overall protein pattern between authentic *O. sinensis* and counterfeits, suggesting that proteomic differences might allow species identification. Using PDQuest 8.0.1 software, 22 distinctive spots were displayed in Fig. 1–I, which were present consistently in all *O. sinensis* samples from different locations, but not found in any fake sample. These spots were excised, digested in gels and analyzed by MALDI-TOF/MS and the results of polypeptide mass fingerprint was obtained by searching matches through Mascot v2.1 engine against

nrNCBI, insect and fungi databases (Table 2). Among the 22 distinctive spots, only spots (9, 15, 20 and 24) were matched against the insect database. These protein species came from the dead bodies of *O. sinensis* ghost moth larvae, suggesting that the host partner was not only a group of nutrients for fungal growth but a part of the species complex (Bhardwaj and Yadav. A common protein extraction protocol for proteomic analysis: 2013; Zhu et al. 2017; Wu et al. 2008). Moreover, spot 19 matched to vacuolar-ATPase, a protein that was previously found to be essential in maintaining morphological changes in *N. crassa*, potentially through maintaining cellular homeostasis (Bowman et al. 2000). Additionally, spot 13 matched to heat shock protein, which was found to be played a crucial role in fruiting body and sexual development (Omeara and Cowen 2014). Spot 3 matched to the hypothetical protein OCS_01884 (gil531865517) in the NCBI protein database, which was also identified in Shiwei Zhang's study (Zhang et al. 2016). Cyanate hydratase (spot 4), nicotinate phosphoribosyl transferase (spot 5) and glycerol-3-phosphate dehydrogenase (spot 8) were abundant in all *O. sinensis* samples from different production regions, but were not found in any counterfeit samples, suggesting these proteins might be the ideal marker candidates for the authentication of *O. sinensis* as well as providing insights to activities unique to this fungus.

Preparation of marker proteins

Three authentic *O. sinensis* samples and three counterfeit samples were examined by AG-PAGE. The protein profiles between *O. sinensis* and counterfeits showed clearly different (Fig. 2). The matching ratios were $38 \pm 0.64\%$ between *O. sinensis* and *C. liangshanensis*, and $57 \pm 0.39\%$ between *O. sinensis* and *C. gunnii*. One distinctive band was observed and isolated from unstained lanes of authentic *O. sinensis* samples for HPLC. The result showed that the purity of the isolated protein was high ($> 90\%$) (Fig. 3). Then, using ESI-Q-TOF/MS, the isolated peptide was identified to be "FEQIAQHIGR", with the coverage of 16%. Using nrNCBI database, the isolated protein matched with cyanate hydratase (gil751836131), which is involved in N-metabolism and N-cycling (Palatinszky et al. 2015). The peptide also matched with spot 4 in 2-D gels, and was found in all authentic *O. sinensis* samples. We, therefore, termed this peptide as IP4. Based on amino acid sequence blast in NCBI, the homolog sequence of IP4 was also found in *C. militaris*. However, our study showed that no corresponding spot was detected in all counterfeit samples, indicating that the content of IP4 may be very low in *C. militaris* or other counterfeits tested. Moreover, the identity of the amino acids is 46% between *O. sinensis* and *C. militaris*, suggesting that IP4 is not evolutionarily conserved and

Table 2 Identification of characteristic protein spots from 2-DE gels of *O. sinensis* by MALDI-TOF-TOF/MS

Spot no.	MW (kDa)	pI	NCI database accession no.	Protein name	% Sequence coverage
1	32.63	4.52	gil398364447	F-actin-capping protein β subunit	2
2	43.05	5.89	gil162462772	Polycomb group protein FIE2 OS	4
3	11.84	5.61	gil531865517	Hypothetical protein OCS_01884	68
4	17.78	5.93	gil751836131	CYANATE hydratase OS	16
5	45.71	6.36	gil751841486	Nicotinate phosphoribosyl transferase OS	2
6	43.85	9.21	gil531862512	Malate dehydrogenase	35
8	34.08	5.05	gil686634833	Glycerol-3-phosphate dehydrogenase	6
9	36.13	4.50	gil283945482	Nuclear excision repair protein Rad 23	4
10,17	39.62	5.51	gil3696	Fructose-bisphosphate aldolase	≥ 15
13	61.945	5.73	gil346323592	Heat shock protein 60 (Antigen HIS-62) OS	15
14,16	62.48	5.02	gil332313314	Carboxypeptidase Y homolog A OS	≥ 3
15	38.95	5.48	gil54036194	3-isopropylmalat-e dehydrogenase OS	2
18	66.73	5.36	gil471916648	Heat shock protein SSB	1
19	66.37	5.37	gil346325359	Vacuolar ATP synthase catalytic subunit A	2
20	22.64	5.62	gil480546539	Elongation factor 2	11
21	37.81	5.75	gil183175030	tRNA-specific 2-thiouridylase MnmA	3
22	31.44	8.59	gil531864346	Glyceraldehyde/Erythrose phosphate dehydrogenase family	33
23	28.27	6.44	gil8698689	Small G-protein GP1p	29
24	51.23	5.60	gil346323366	Rab GDP-dissociation inhibitor	15

MW molecular weight, kDa kilo dalton

Fig. 2 Alkaline native gradient PAGE map of 120 µg total protein, 5–15% separation gel, proteins extraction from authentic *O. sinensis* samples collected from three locations and two counterfeit samples. lane1, 4 and 7, corresponds to sample codes: OS1, OS2 and OS3; lane 2 and 5, corresponds to sample code: CL; lane 3 and 6, corresponds to sample code: CG

Fig. 3 HPLC chromatograms of the purified IP4 solution recorded at 280 nm. Column: Agilent 300A C18 (250×4.6 mm, 5 µm; Agilent, Inc, USA), a mobile phase consisting of acetonitrile containing 0.1% trifluoroacetic acid solution and water containing 0.1% trifluoroacetic acid, Flow rate: 1.0 ml/min, Input volume: 100 µL, Peak eluted at 3.5 min

might be responsible for variation in immune response. Therefore, IP4 was subsequently used as a putative target to identify and authenticate *O. sinensis*.

Production of anti-IP4 polyclonal antibody and detection of the specificity

Three male BALB/c mice were immunized with the purified IP4 solution and the titer of the anti-sera approximately reached 1:128 by DID. The analysis of the specificity was assayed by indirect ELISA. The result showed a positive signal for IP4 and the immune sera, while no signals were observed in negative controls. So, we conclude that the anti-IP4 polyclonal antibody was specific to IP4.

Development and evaluation of the indirect ELISA

To the best of our knowledge, immunoassay hasn't been developed to authenticate *O. sinensis*. The characteristic protein (IP4) would be used as an indicator, which facilitates the development of a fast and simple immunoassay. The proposed protocol based on an indirect-ELISA quantification of a protein marker was first established. The indirect-ELISA conditions were optimized as followed: wells were coated with 100 µL antigen (0.5 mg/ml) in NBS carbonate buffer (0.01 mol/l, pH 9.6) and incubated at 37 °C for 5 h, then overnight at 4 °C, 200 µL 1% BSA-PBS blocking buffer were added and incubated at 37 °C for 2 h, the suitable dilution of anti-IP4 anti-sera was 1:128 and that of HRP-labeled goat anti-mouse IgG was 1:3000. TMB was added to the wells as substrate and incubated for 20 min.

To evaluate the feasibility of the indirect ELISA, three authentic *O. sinensis* and three counterfeit samples were examined. The serially diluted IP4 was assayed by our established indirect ELISA. The linear range of the standard curve was 0.1 to 0.6 mg/ml, and the squared correlation coefficient (R^2) was 0.9916. The results of our indirect ELISA assay showed that the P/N ratios of three authentic *O. sinensis* samples were over 8, whereas the P/N ratios of three counterfeits were less than 1 (Table 3), indicating that the proposed protocol based on ELISA quantification of IP4 has a great potential in authentication and quality assessment of *O. sinensis* commercial products. Our future endeavor is

Table 3 ELISA analysis of samples

Sample	OD ₄₅₀			Content of IP4 (mg/ml)
	P	N	P/N	
OS1	2.777	0.284	9.778	0.492
OS2	2.382	0.277	8.599	0.396
OS3	2.484	0.284	8.746	0.421
CL	0.335	0.357	0.938	0
CG	0.312	0.333	0.937	0
CM	0.399	0.408	0.978	0

P sample OD₄₅₀, N negative control OD₄₅₀

to detect more distinctive and specific protein markers, followed by the production of monoclonal antibody to enhance the sensitivity and specificity of this indirect ELISA method.

Conclusion

In this study, proteomic protocol was used to identify characteristic proteins in *O. sinensis*. Among them, one abundant and specific protein, cyanate hydratase (IP4), was isolated and purified. Using the anti-sera against IP4, a new indirect ELISA method for the authentication of *O. sinensis* was developed. The results indicated that proteomic protocol was reliable in identifying protein marker in *O. sinensis*, and the proposed protocol based on ELISA quantification has a great potential in authentication of *O. sinensis*, quality assessment of this fungus in food, healthcare products and also TCM.

Acknowledgements This study was supported by the Natural Sciences Foundation of China Science (81373920, 30801522), the Foundation of Administration of TCM of Sichuan, the Science Foundation of Administration of Science and Technology Bureau of Chengdu, and the Foundation of National Engineering Research Center of Solid-State Brewing.

Author contributions Jinlin Guo and Cheng Peng conceived and designed the experimental. Xinxin Tong and Yixuan Wang participated in proteomic experiment, the relevant data analyses, manuscript writing and revise. Zhengyao Xue participated in proteomic data analyses, this part writing and manuscript revise. Lu Chen and Yi Qiu participated in ELISA experiment. Jing Cao participated in sample collecting, preparing and treating. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Azevedo PZ, Sylvestre TF, de Souza R et al (2014) Evaluation of the double agar gel Immunodiffusion test and of the enzyme-linked immunosorbent assay in the diagnosis and follow-up of patients with chronic pulmonary aspergillosis [J]. Plos one 10(8):e0134841
- Bhardwaj J, Yadav SK (2013) A common protein extraction protocol for proteomic analysis horse gram a case study [J]. Am J Agric Biol Sci 8(4):293–301
- Bowman EJ, Kendle R, Bowman BJ (2000) Disruption of vma-1, the gene encoding the catalytic subunit of the vacuolar H(+)-ATPase, causes severe morphological changes in *Neurospora crassa* [J]. J Biol Chem 275:167–176
- Dong C, Guo S, Wang W et al (2015) Cordyceps industry in China [J]. Mycology 6(2):1–9
- En-Hua Xia D-R, Yang J-J, Jiang et al (2017) The caterpillar fungus, *Ophiocordyceps sinensis*, genome provides insights into highland adaptation of fungal pathogenicity [J]. Sci Rep 11(1):1806–1817
- Grog A, Weiss W, Dumn MJ (2004) Current two-dimensional electrophoresis technology for proteomics [J]. Proteomics 4(12):3665–3685
- Hankun Hu, LingXiao B, Zheng et al (2015) Identification of chemical markers in *Cordyceps sinensis* by HPLC-MS/MS [J]. Anal Bioanal Chem 407(26):8059–8066
- Heinrich M, Anagnostou S (2017) From pharmacognosia to DNA-based medicinal plant authentication—pharmacognosy through the centuries [J]. Planta Med. <https://doi.org/10.1055/s-0043-108999>
- Hunag yi (2003) Hua chao. Application of electro elution into dialysis bag in protein recovery and purification [J]. J Nanjing Mil Med Coll 25(1):33–34
- James M, Harnly P, Chen J, Sun et al (2016) Comparison of flow injection MS, NMR, and DNA sequencing: methods for identification and authentication of Black Cohosh (*Actaea racemosa*) [J]. Planta Medica 82(3):250–262
- Jin GS, Wang XL, Li Y (2013) Development of conventional and nested PCR assays for the detection of *Ophiocordyceps sinensis* [J]. J Basic Microbiol 53(4):340–347
- Jinlin Guo X, Liu K, Kanari (2012) Economic aspects of harvesting and trading the Chinese Caterpillar Fungus *Ophiocordyceps sinensis* and Southern Schisandra *Schisandra sphenanthera* in China's Upper Yangtze Ecoregion [J]. TRAFFIC Bulletin 24(1):15–24
- John E, Coligan (2007) Current Protocols in Protein Science CPPS [M]. Science press, Beijing
- Katayama H, Nagasu T, Oda Y (2001) Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [J]. Rapid Commun Mass Spectrom 15(16):1416–1421
- Kruger NJ (2009) Methods in molecular biology [M]. New York, United States: Humana Press, pp 461–463
- Li GJ, Fu FL (2007) Two dimensional electrophoresis and extraction of total protein of maize [J]. J Maize Sci 14(6): 100–103
- Lianxian Guo X, Xu F, Liang et al (2015) Morphological observations and fatty acid composition of indoor-cultivated *Cordyceps sinensis* at a high-altitude laboratory on Sejila Mountain, Tibet [J]. PLoS One 10(5):e0126095
- Liu H, Hu H, Chu C et al (2011) Morphological and microscopic identification studies of *Cordyceps* and its counterfeits [J]. Acta Pharmaceutica Sinica B 1(3):189–195
- Omeara TR, Cowen LE (2014) Hsp90-dependent regulatory circuitry controlling temperature-dependent fungal development and virulence [J]. Cell Microbiol 16(4):473–481
- Palatinszky M, Herbold CW, Jehmlich N et al (2015) Cyanate as an energy source for nitrifiers [J]. Nature 524(7563):105–108
- Qian G, Pan GF, Guo JY (2012) Anti-inflammatory and antinociceptive effects of cordymin, a peptide purified from the medicinal mushroom *Cordyceps sinensis* [J]. Nat Prod Res 26(24):2358–2362
- Qian Z, Li S (2017) Analysis of *Cordyceps* by multi-column liquid chromatography. Acta Pharm Sin B 7(2):202–207
- Ren yan (2013) The study of the species and protein components of precious traditional Chinese Medicine *Cordyceps* [D]. Chengdu. Chengdu university of TCM
- Song chunhua, Xi yuanling, Zhang meixi (2007) Establishment of two-dimensional proteome map of *Shigella flexneri* [J]. J Zhengzhou Univ Med Sci 42(2): 316–318
- Tuli HS, Sandhu SS, Sharma AK (2014) Pharmacological and therapeutic potential of *Cordyceps* with special reference to *Cordycepin* [J]. Biotech 4(1):1–12
- Wang ZB, Li N, Wang M et al (2013) Simultaneous determination of nucleosides and their bases in *Cordyceps sinensis* and its substitutes by matrix solid-phase dispersion extraction and HPLC [J]. J Sep Sci 36(14):2348–2357
- Wang Hao Z Zhi-guo, Chang Yan-zhong, et al. Isolation and quantification of main milk proteins in dairy products with reverse-phase HPLC [J]. Food Sci, 2009, (24):376–380

- Wu YS, Zhou DL, Yan D, Ren YS et al (2008) HPLC fingerprint analysis of cordyceps and mycelium of cultured Cordy [J]. Chin J Chin Materia Medica 33(19):2212–2214
- Xiang L, Song J, Xin T et al (2013) DNA barcoding the commercial Chinese caterpillar fungus [J]. FEMS Microbiol Lett 347(2):156–162
- Xu J, Huang Y, Chen X et al (2016) The mechanisms of pharmacological activities of *Ophiocordyceps sinensis* Fungi [J]. Phytotherapy Research 30(10):1572–1583
- Yang FQ, Li DQ, Feng K et al (2010) Determination of nucleotides, nucleosides and their transformation products in Cordyceps by ion-pairing reversed-phase liquid chromatography-mass spectrometry [J]. J Chromatogr A 1217(34):5501–5510
- Zhang B, Barekati Z, Kohler C et al (2010) Proteomics and Biomarkers for Ovarian Cancer [J]. Ann Clin Lab Sci 40(3):218–225
- Zhang L, Dai X, Xu F et al (2012a) Preparation of imidazole-functionalized silica by surface-initiated atom transfer radical polymerization and its application for hydrophilic interaction chromatography [J]. AnalBioanal Chem 404(5):1477–1484
- Zhang Y, Li E, Wang C et al (2012b) *Ophiocordyceps sinensis*, the flagship fungus of China: terminology, life strategy and ecology [J]. Mycology 3(1):2–10
- Zhang S, Zhang YJ, Liu XZ et al (2013) On the reliability of DNA sequences of *Ophiocordyceps sinensis* in public databases [J]. J Ind Microbiol Biotechnol 40(3):365–378
- Zhang S, Lai X, Li B et al (2016) Application of differential proteomic analysis to authenticate ophiocordyceps sinensis [J]. Curr Microbiol 72(3):337–343
- Zhu xiaohui (2014) Sheng deqiao, Kang le. the preparation and application of mouse DEAF1 polyclonal antibody [J]. Biotechnol Bull 22(5):197–201
- Zhu J-S, Guo YL, Yao YS et al (2017) Maturation of Cordyceps sinensis associates with co-existence of Hirsutella sinensis and Paecilomyces hepiali DNA and dynamic changes in fungal competitive proliferation predominance and chemical profiles [J]. J Fungal Res 5(4):214–224