INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1162/2020/23–4–820–828
DOI: 10.17957/IJAB/15.1357
http://www.fspublishers.org

Full Length Article

iTRAQ-Based Quantitative Differentially Expressed Proteins of Wolfberry (*Lycium barbarum*) from Different Habitats in China

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Received 18 July 2019; Accepted 24 December 2019; Published 13 February 2020

Abstract

This study was to research the proteome differences of 'Ningqi No. 1' wolfberry (*Lycium barbarum* L., also known as 'goji') from Zhongning Ningxia and other four habitats in China (Jinghe, Xinjiang, Urat Front Banner, Inner Mongolia, Guazhou, Gansu, and Delingha, Qinghai) by iTRAQ and 2D LC-MS/MS. A total of 4852 proteins were identified with at least one unique peptide. And 166 (Inner Mongolia/Ningxia), 446 (Xinjiang/Ningxia), 1015 (Gansu/Ningxia) and 996 (Qinghai/Ningxia) DEPs showed a significant ($P < 0.05$) change (≥1.2 or ≤0.83) in relative abundance. Gansu/Ningxia and Qinghai/Ningxia have more DEPs than Xinjiang/Ningxia and Inner Mongolia/Ningxia. Bioinformatic and functional analysis showed that DEPs were mainly bound up with GO (gene ontology) functional items, such as 'metabolic process', 'cellular process' and 'single−organism process'; DEPs were mainly involved in the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway of 'carbon metabolism', followed by 'biosynthesis of amino acids' and 'protein processing in endoplasmic reticulum'. These results suggested that wolfberry in Ningxia had little difference with Inner Mongolia and Xinjiang, but had great difference with Gansu and Qinghai, and their differences were mainly in 'metabolic progress'. This research may supply a new visual angle to understand the differences of wolfberry from different habitats.

Keywords: Wolfberry (*Lycium barbarum* L.); GO (Gene Ontology); KEGG (Kyoto Encyclopedia of Genes and Genomes); Differentially expressed proteins (DEPs)

Introduction

Wolfberry (*Lycium barbarum* L., also known as 'goji') is a kind of traditional Chinese medicine, belonging to the genus *Lycium* in Solanaceae. Wolfberry contains lots of bioactive compounds and trace elements, and has many medicinal functions (Zhou *et al*. 2018). In traditional Chinese medicine, wolfberry was used as a mild tonic (Potterat 2009) and reported to reduce blood sugar and lipids and hypertension, and exhibit other bioactivities including anti-aging, immune regulation, anti-cancer, anti-fatigue, protection of liver and kidneys, and promotion of male fertility (Luo *et al*. 2004; 2006; Li *et al*. 2007; Amagase and Farnsworth 2011; Li *et al*. 2011). And it also has shown that wolfberry can increase retinal ganglion cell survival after rat partial optic nerve transection (Li *et al*. 2019). As a result, wolfberry has become one of the most popular fruits in the global marketplace, and is also processed into different products, such as wolfberry juice and wine (Benzie *et al*. 2006; Ren *et al*. 2018). At the same time, some researches have combined wolfberry with other food ingredients. Can made of chrysanthemum flower (*Chrysanthemum morifolium* cv. Hangju) and wolfberry can resist oxidation and inflammatory (Zhang *et al*. 2019a).

Wolfberry is mainly produced in the northwest of China, mainly including Ningxia, Gansu, Inner Mongolia, Xinjiang and Qinghai Provinces. Of these, the quality of the wolfberry in Zhongning County, Ningxia Province is the best, and Zhongning County is known as the birthplace of *Lycium barbarum* L. in the world, with more than 600 years history of wolfberry cultivation (Zhang *et al*. 2017). The Ministry of Agriculture, China formally approved the registration and protection of geographical indications for 'Zhongning wolfberry' as one of the agricultural products on January 10, 2017. In recent years, a number of new varieties have been produced through screening of natural dominant mutants and one of the main varieties widely cultivated in the northwest of China is 'Ningqi No. 1'. However, due to the different geographical sources, cultivation methods, light intensities, temperatures, precipitations, soils and other environmental factors, wolfberries from different habitats show great differences in medical functions (Meng *et al*. 2019). At present, the researches on wolfberry from different habitats mainly focused on the determination of the contents of active compounds (Yin and Dang 2008; Wang *et al*. 2010), fingerprint evaluation (Peng *et al*. 2004; Lu *et al*. 2014; Liu *et al*. 2015), cultivation technology (Zhang *et al*. 2013), etc.

To cite this paper: Kou T, Y Niu, Y Fan, J Chen, L Han (2020). iTRAQ-based quantitative differentially expressed proteins of wolfberry (*Lycium barbarum*) from different habitats in China. *Intl J Agric Biol* 23:820–828

Proteomics had been applied in many fields. The effect of different concentrations of nitrogen on different plants was studied by proteomics (Tan *et al*. 2017; Xiong *et al*. 2019; Tang *et al*. 2019), and proteomics was combined with other omics to analyze the effects of different culture conditions on crops (Guzman-Albores *et al*. 2019), as well as the tolerance and molecular defense mechanisms of plants under various stresses (Wei *et al*. 2019a; Jia *et al*. 2019; Almutrairi 2019). Label-free proteomic and untargeted metabolomic analysis were used to characterize and differentiate ginger samples from China and Ghana, and a total of 180 significantly different proteins were found, which could be the underlying cause of the intraspecific differences in ginger samples (Yin *et al*. 2017). The protein expression level of different varieties of kiwifruit (*Actinidia* Lindl.) were compared by proteomics, which was helpful to understand the metabolic pathway and biological process of different kiwifruit flesh color changes (Lin *et al*. 2017). The molecular mechanism of tobacco response to different climatic environments was preliminarily clarified by analyzing the protein expression level of the same variety from different habitats (Cai *et al*. 2013). However, it has not been found that the geographical differences of wolfberry from the perspective of proteins. It is essential to study wolfberry by proteomics in order to further determine the differences of wolfberry from different habitats in China.

In this study, the quantitative proteomics of wolfberry from five habitats was studied by iTRAQ and 2D LC-MS/MS, and the differences of wolfberry proteome expression levels between Zhongning, Ningxia and other four habitats (Jinghe, Xinjiang, Urat Front Banner, Inner Mongolia, Guazhou, Gansu, and Delingha, Qinghai) were analyzed respectively. Through the bioinformatics analysis, we obtained the protein information of wolfberry from different habitats. The purpose of this study was to determine the main differences of wolfberry proteins in different habitats, to provide a reference for the study of synthesis and accumulation of active compounds in wolfberry, and to help clarify the quality formation mechanism of wolfberry.

Materials and Methods

Sample sources

Wolfberry (variety 'Ningqi No. 1') fruits were collected from Ningxia, Xinjiang, Inner Mongolia, Gansu and Qinghai, China from July 20 to August 10, 2017. According to the harvest time of different habitats, we harvested fresh fruits which could represent the characteristics of the local wolfberry. For the sake of minimizing the influence of the field management on the quality of wolfberry, the factors such as tree age, density and pruning conditions were fully taken into account in sampling. 'Ningqi No. 1' wolfberries with 6–10 years old were randomly selected from each habitat. In the garden, we selected three normal plants grown continuously in the middle of the garden, and the mixed fresh fruits of the wolfberry were collected from all parts of the trees. After harvesting, the fruits were frozen in liquid nitrogen immediately, and stored at -80^{o}C for further analysis.

Protein extraction

Trichloroacetic acid (TCA)/acetone and SDT lysis method were used to extract the total protein of wolfberry (Zhu *et al*. 2014). 5% (m/v) TCA/acetone (1:9) was added into the finely powder wolfberry and mixed it with vortex. The mixture was precipitated at -20^{o}C for 4 h. After centrifuged (6,000 × g, 4^{o}C 40 min), the precipitate was air dried. 30% (m/v) of SDT buffer was added into 20 μg powder, then mixed and boiled for 5 min. The homogenate was sonicated for 80 W, 10 s, intermittent for 15 s, 10 times and then boiled for 15 min. After centrifuged (14,000 × g, 40 min), the supernatants were filtered by 0.22 μm filters. Then the BCA Protein Assay Kit (Bio-Rad, USA) was used to determine the protein content. And the samples were stored at -80ºC.

SDS-polyacrylamide gel electrophoresis (PAGE)

The experimental steps were same as previously mentioned (Li *et al*. 2017). 20 μg of proteins for each sample were added to 5 × loading buffer (10% SDS, 0.5% Bromophenol Blue, 50% glycerol, 500 m*M* DTT, 250 m*M* Tris-HCL, pH 6.8), and then boiled in water for 5 min. After that, 5% stacking gels and 12.5% resolving gels (Seebio, Shanghai, China) were used for SDS-PAGE analysis of the samples respectively (14 mA, 90 min), and the protein bands were visualized by Coomassie Blue R-250 (Invitrogen) staining.

Protein digestion

The filter-aided sample preparation (FASP) procedure was used to digest the protein (Wisniewski *et al*. 2009). 200 μg of wolfberry protein sample was dissolved in 30 μL SDT buffer (4% SDS, 100 m*M* DTT and 150 m*M* Tris-HCl, pH 8.0), incubated in boiling water for 5 min and cooled them down to the room temperature. Then the experimental steps were same as previously described (Chen *et al*. 2017). The sample was added with 200 μL UA buffer (8 M urea, 150 m*M* Tris-HCl, pH 8.0), placed in a 10 kD filter (Sartorius, German), then centrifuged (14,000 × g, 15 min). This step was repeated once. The sample was added with 100 μL IAA buffer (100 m*M* IAA in UA), incubated for 30 min in darkness, then centrifuged (14,000 × g, 15 min). Then filters were washed three times with 100 μL UA buffer, and washed twice with 100 μL Dissolution buffer (DS buffer). 40 μL of trypsin (Promega, Madison, WI, USA) buffer (4 μg trypsin in 40 μL DS buffer) was added, then the samples were digested overnight at 37℃. After centrifuged (14,000 × g, 15 min), the BCA Protein Assay Kit was used to determine the protein content.

iTRAQ labeling

According to the manufacturer's instructions, 100 μg peptide mixture of each sample was labeled using the iTRAQ reagent (Applied Biosystems, Framingham, MA, U.S.A.). And the protein samples were labeled with 113 (Zhongning, Ningxia), 114 (Jinghe, Xinjiang), 115 (Urat Front Banner, Inner Mongolia), 116 (Guazhou, Gansu) and 117 (Delingha, Qinghai) respectively.

Strong cation exchange (SCX) chromatography

iTRAQ labeled peptides were fractionated using the AKTA Purifier system (GE Healthcare, Litter Chalfont, United Kingdom) by SCX chromatography. Recombined the dried peptide mixtures, acidified it with buffer A (10 m*M* KH_2PO_4 in 25% of ACN, pH 3.0) and loaded it into a Poly SULFOETHYL 4.6 × 100 mm column (5 µm, 200 Å, Poly LC Inc., MD, USA). The peptide was eluted with buffer B (500 m*M* KCl, 10 m*M* KH_2PO_4 in 25% of ACN, pH 3.0) at a flow rate gradient of 1 mL/min. The linear gradient of buffer B absorbance value and follow-up steps could be found in the previous study (0–8% for 22 min, 8–52% during 22–47 min, 52–100% during 47–50 min, 100% during 50–58 min, and the buffer B was reset to 0% after 58 min) (Lin *et al*. 2017). The elution was monitored at 214 nm, the components were collected every minute and a total of 30 fractions were collected. Then each group of samples was divided into 3 portions, the collected fractions were desalted on C18 Cartridges and concentrated by vacuum centrifugation.

LC-MS/MS analysis

Every sample was separated using a HPLC liquid phase system Easy nLC (Thermo Fisher Scientific, Odense, Denmark). 0.1% formic acid was Buffer A, 0.1% formic acid (84% acetonitrile) was Buffer B, and the column was equilibrated with 95% Buffer A. The sample was loaded into a loading column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nano Viper C18) from an autosampler and separated by an analytical column (Thermo Scientific Easy Column, 10 cm, ID 75 μm inner diameter, 3 μm resin, C18-A2) at a flow rate of 300 nL/min by IntelliFlow technology. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA, USA), and it was coupled to Easy nLC. And the situation was same as previously described (Wang *et al*. 2013).

Database search and protein identification and quantification

The identification and quantification of mass spectrometry were performed by MASCOT engine (Matrix Science, London, U.K., version 2.2) embedded into Proteome Discoverer 1.4. The MS/MS data were searched against the database for protein identification and quantification, and the criteria were as followed: uniprot_Solanoideae_139867_20170221 protein database (downloaded February 2017, 818,444 sequences). The MASCOT parameters were set same as previously mentioned (Wang *et al*. 2013).

The reported data were based on at least one unique peptide with 99% confidence for protein identification as determined by false discovery rate (FDR)⩽1% (Yuan *et al*. 2018). There was at least one unique peptide in each successfully identified protein. All peptide ratios were standardized with the median protein ratio, which should be 1. Up- or down-regulated proteins were determined with a 1.2-fold cutoff, and a $P < 0.05$ (Chu *et al*. 2015; Miao *et al*. 2015). The protein ratio was further analyzed by Student's *t*-test, and the statistical package was Perseus1.3.0.4. And the 'Ningqi No. 1' wolfberry in Zhongning, Ningxia was considered as a relative quantitative reference.

Bioinformatic and statistical analysis

We performed GO annotation (http://www.geneontology.org/) and the KEGG pathway (http://www.genome.jp/kegg/) annotation on DEPs using Blast2GO (Gotz *et al*. 2008) program and KAAS (KEGG Automatic Annotation Server) software (Moriya *et al*. 2007) respectively. And then Fisher's Exact Test was used to perform an enrichment analysis of GO annotations and KEGG pathway annotations for the DEPs.

Results

Analysis of SDS-PAGE in protein samples of wolfberry

As shown in Fig. 1, each strip was distributed between 14.4 and 116 kD, and the molecular weights of most proteins were between 18.4 and 25 kD. The distribution patterns of strips among different samples were similar. The results preliminarily showed that the proteins extracted from wolfberry met the requirements of iTRAQ technology, and thus could be further analyzed by protein labeling and chromatography.

Protein identification and quantification

Three repeated experiments were carried out to compare the wolfberry proteome by LC-MS/MS technology. As shown in Table 1, total of 818,444 LC MS/MS spectra were matched to known spectra; among them, the number of matched spectra was 60,795, and the utilization rate of the spectrum was 7.43%; a total of 16,384 and 12,375 unique peptides were obtained. These peptides could identify 4,852 proteins of the wolfberry.

Mascot software was used to visualize the identified proteins. Most of the identified proteins (72.88%) had molecular weights ranging from 10–20 kD (661 proteins),

Table 1: Statistics of the protein identification results

No.	Total spectra	Spectra (PSM)	Peptides	Unique peptides	Protein groups
1	276883	21380	11360	8877	4093
2	268829	19591	10657	8350	3903
3	272732	19824	10740	8410	3903
1,2,3, Combination and results	818444	60795	16384	12375	4852

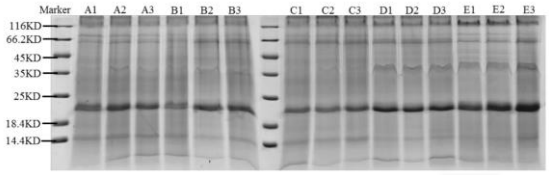

Fig. 1: SDS-PAGE analysis. Markers represent the protein standards. A/B/C/D/E represent the proteins in wolfberry from Zhongning, Ningxia, Jinghe, Xinjiang, Urat Front Banner, Inner Mongolia, Guazhou, Gansu and Delingha, Qinghai respectively, and 1/2/3 represents three repeated experiments

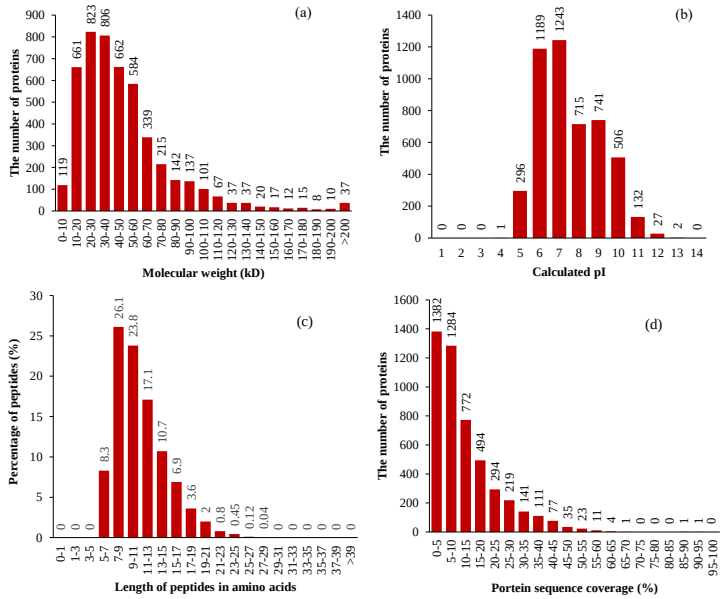

Fig. 2: Characteristics of the peptides. **(a)** Distribution of the identified proteins with different molecular weights (kD). **(b)** Distribution of the identified proteins with different isoelectric points. **(c)** Distribution of the identified proteins with different peptide length. **(d)** Distribution of the identified proteins with protein sequence coverage (%)

20–30 kD (823 proteins), 30–40 kD (806 proteins), 40–50 kD (662 proteins), or 50–60 kD (584 proteins) (Fig. 2a). As shown in Fig. 2b, the isoelectric points of most of the identified proteins (90.56%) were 6–10 (4,394 proteins). In the identified proteins, the number of amino acids was mainly 5–19, of which 7–11 were the most (Fig. 2c). As shown in Fig. 2d, the identified proteins had high peptide coverage.

In comparison group with the wolfberry samples in Ningxia, a total of 1,437 DEPs were separated under the three biological replications. As shown in Fig. 3a, of the 1,437 DEPs, 160 (286), 55 (111), 438 (528) and 498 (517) proteins were up-regulated (down-) in Xinjiang/Ningxia, Inner Mongolia/Ningxia, Gansu/Ningxia and Qinghai/Ningxia respectively.

We could find that the number of DEPs was different between Ningxia and other habitats: Ningxia/Qinghai had the largest number of DEPs, followed by Gansu/Ningxia and Xinjiang/Ningxia and the least DEPs were found in Ningxia/ Inner Mongolia. In addition, we could find that the number of up-regulated DEPs was always less than the down-. Meanwhile, we used Venn diagrams to study the overlap of DEPs among the four comparison groups. As shown in Fig. 3b, we found 380 DEPs in one group, 452 DEPs were found in any two groups, 258 DEPs were found in any three groups and 56 DEPs were found in all three groups. It was found that the number of DEPs was different between Ningxia and other habitats: Ningxia and Qinghai had the largest number of DEPs, followed by Gansu and Xinjiang, and the least DEPs were found in Ningxia and Inner Mongolia.

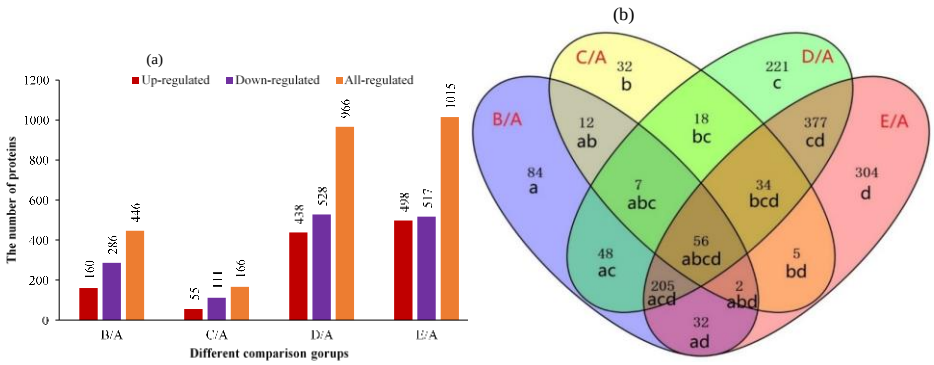

Fig. 3: Differential expression of proteins. **(a)** The number of up-regulated, down-regulated and total DEPs in each group. **(b)** The overlap in total DEPs among the four groups. A/B/C/D/E notes were shown in Fig. 1

Bioinformatics analysis

GO functional classification: The functional classification of all DEPs was determined by analyzing their biological process (BP), molecular function (MF) and cellular component (CC). The comparison between Ningxia and Xinjiang is shown in Fig. 4a, DEPs were classified into 47 functional groups, of which BP accounted for 20 GO terms, MF accounted for 11 Go terms, and CC accounted for 16 GO terms. The GO terms included 'developmental process' (P = 0.01147), 'growth' (P = 0.03839), 'reproduction' (P = 0.00693), 'multicellular organismal process' (P = 0.01608), 'reproductive process' (P = 0.00617) and 'response to stimulus' (P = 0.01123).

As depicted in Fig. 4b, in the comparison group of Ningxia and Inner Mongolia, DEPs were classified into 41 functional groups, of which BP accounted for 19 GO terms, MF accounted for 9 GO terms, and CC accounted for 13 GO terms. The GO terms included 'detoxification' (P = 0.00810), 'antioxidant activity' (P = 0.00689) and 'membrane' (P = 0.02226).

In the comparison group of Ningxia and Gansu (Fig. 4c), DEPs were classified into 49 functional groups, of which BP accounted for 21 GO terms, MF accounted for 12 GO terms, and CC accounted for 16 GO terms. The GO terms included 'metabolic process' (P = 0.04212) and 'structural molecule activity' (P = 0.00337).

In the comparison group of Ningxia and Qinghai (Fig. 4d), DEPs were classified into 48 functional groups, of which BP accounted for 21 GO terms, MF accounted for 11 GO terms, and CC accounted for 16 GO terms. The GO terms included 'detoxification' (P = 0.03390), 'antioxidant activity' (P = 0.02454) and 'structural molecule activity' (P = 0.00637).

In these 4 groups, 'metabolic process', 'cellular process' and 'single−organism process' were the most important terms among biological processes, 'catalytic activity and 'binding' were the most important terms among molecular functions, and 'cell', 'cell part' and 'organelle' were the most important terms among cellular components.

KEGG pathway analysis: Different proteins usually work together to perform their biological functions and we can use a pathway-based analysis to learn more about the biological functions of proteins. The comparison between Ningxia and Xinjiang is shown in Fig. 5a, the most aplenty DEPs in KEGG pathway were bound up with 'carbon metabolism' (10 up-regulated, 17 down-regulated), and the main pathways in the KEGG enrichment analysis were 'peroxisome' (P = 0.01815).

As shown in Fig. 5b, in the comparison group of Ningxia and Inner Mongolia, DEPs with KEGG pathway mostly affected protein processing in 'endoplasmic reticulum' (1 up-regulated, 10 down-regulated), and the main pathways in the KEGG enrichment analysis were 'protein processing in endoplasmic reticulum' (P = 0.00761), 'purine metabolism' (P = 0.04420) and 'phenylpropanoid biosynthesis' (P = 0.04708).

In the comparison group of Ningxia and Gansu (Fig. 5c), the most aplenty DEPs in KEGG pathway were bound up with 'carbon metabolism' (27 up-regulated, 31 down-regulated), and the main pathways in the KEGG enrichment analysis were 'ribosome' (P = 0.00018), 'carbon metabolism' (P = 0.04189), 'biosynthesis of amino acids' (P = 0.02000), 'glycolysis / Gluconeogenesis' (P = 0.02571), 'carbon fixation in photosynthetic organisms' (P = 0.00164), 'glyoxylate and dicarboxylate metabolism' (P = 0.0204), '2-Oxocarboxylic acid metabolism' (P = 0.00724), 'peroxisome' (P = 0.009062) and 'fructose and mannose metabolism' (P = 0.02895).

In the comparison group of Ningxia and Qinghai (Fig. 5d) the most aplenty DEPs in KEGG pathway were bound up with 'carbon metabolism' (23 up-regulated, 32 down-regulated), and the main pathways in the KEGG enrichment analysis were '2-Oxocarboxylic acid metabolism' (P = 0.01217), 'protein processing in endoplasmic reticulum' (P = 0.01607), 'phenylpropanoid biosynthesis' (P = 0.04519), 'longevity regulating pathway-multiple species' (P = 0.04141) and 'ascorbate and aldarate metabolism' (P = 0.04350).

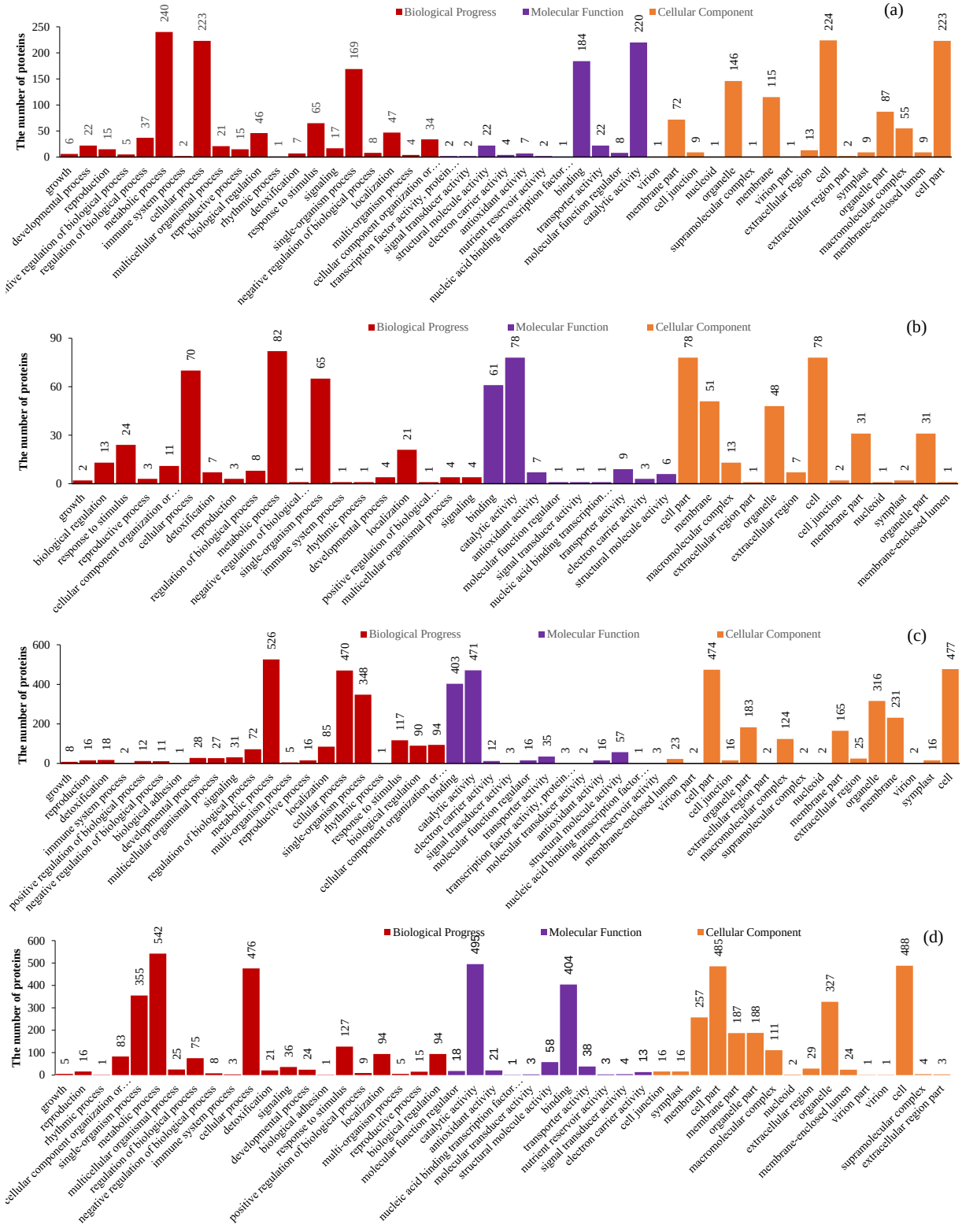

Fig. 4: GO classification of DEPs from different comparison groups. **(a)** Xinjiang/Ningxia. **(b)** Inner Mongolia/Ningxia. **(c)** Gansu/Ningxia. **(d)** Qinghai/Ningxia

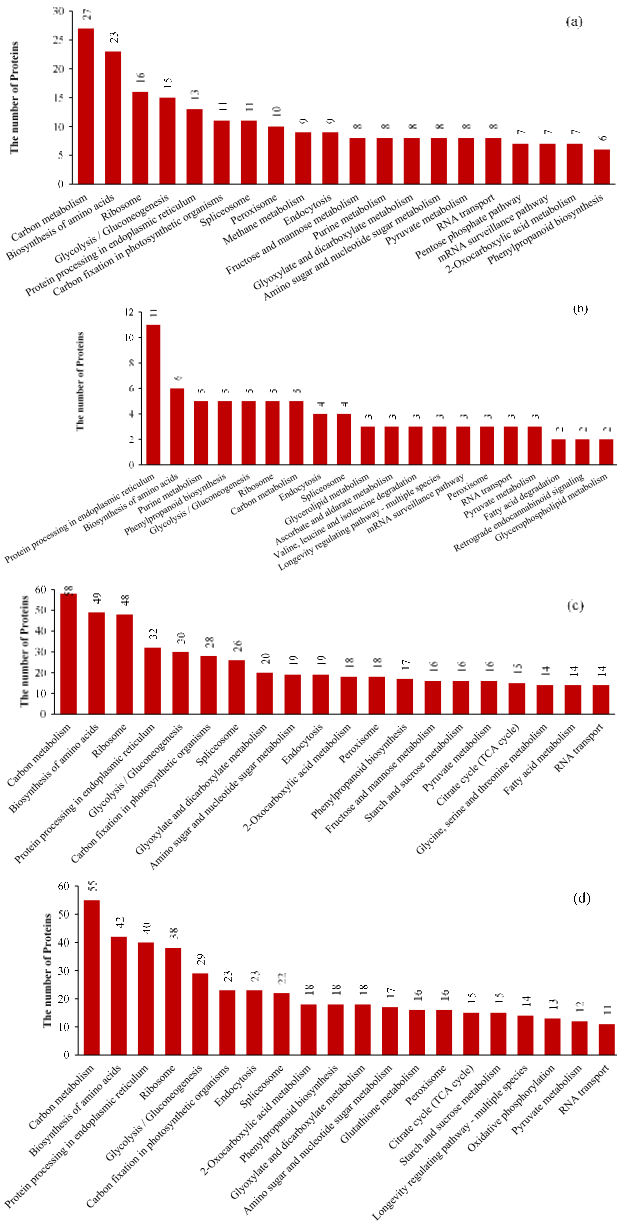

Fig. 5: Kyoto encyclopedia of genes and genomes (KEGG) classification of DEPs from different comparison groups (Top 20). **(a)** Xinjiang/Ningxia. **(b)** Inner Mongolia/Ningxia. **(c)** Gansu/Ningxia. **(d)** Qinghai/Ningxia

Discussion

In this study, proteomics was used to study the protein differences of wolfberry between Zhongning, Ningxia and other different habitats in China. A research has shown that there were no shared DEPs between goat and bovine comparison groups, probably because they are two different species (Wei *et al*. 2019b). But it was found there were some shared DEPs between the four comparison groups in our paper, this could be because they belong to the same

Fig. 6: Distribution of sampling spatial points of wolfberry. A/B/C/D/E represent the proteins in wolfberry from Zhongning, Ningxia, Jinghe, Xinjiang, Urat Front Banner, Inner Mongolia, Guazhou, Gansu and Delingha, Qinghai respectively

wolfberry species 'Ningqi No. 1'. It was found that the results of GO functional annotation analysis of peach fruits produced at different varieties of kiwifruit (Lin *et al.* 2017; Huan *et al*. 2019) were basically consistent with this study, suggesting that our results were credible. Central carbon metabolism was one of the most basic cellular metabolic pathways of all living organisms (Bar-Even *et al.* 2012). Through the KEGG pathway analysis we found that 'carbon metabolism' was the most represented pathway. Among the four groups, one of the most significant differences was 'carbon metabolism' in photosynthetic organisms. Photosynthetic is the decisive factor of sugar synthesis and transportation and connects the environmental and biological factors that regulate fruit development (Chen *et al.* 2002; Hu *et al*. 2019).

And some researches have shown that environment factors are the main causes of wolfberry fruit morphological changes of 'Ningqi No. 1' (Su *et al*. 2015) and the global distribution of wolfberry has obvious regional characteristics, the high temperature, low precipitation and high altitude are the main factors limiting the growth of wolfberry (Amagase and Farnsworth 2011). According to the distribution of wolfberry sampling spatial points (Fig. 6), the altitude of Guazhou, Gansu (2452 meters) and Delingha, Qinghai (2982 meters) are obviously higher than Zhongning, Ningxia (1225 meters), Urat Front Banner, Inner Mongolia (1022 meters) and Jinghe, Xinjiang (332 meters). Precipitation and temperature are usually lower at high altitudes, and the soil factors will be different, too (Haag *et al*. 2019; Zhang *et al*. 2019b). We speculated that the different climate and environmental factors, especially the altitude, may lead to different expression levels of wolfberry proteins.

Although the genome of an organism is usually stable and unchangeable, the expression and composition of proteome has been changing in the process of growth and various physiological processes (Hua *et al*. 2015). Ecological factors in different habitats such as altitude, light intensities, temperatures, precipitations and soil may induce the change of expression products, leading to differential expression of proteins (Cai *et al*. 2013; Yin *et al*. 2017), and accumulation of different secondary metabolites was further induced (Feng *et al*. 2018; Fu *et al*. 2019). Wolfberry fruit is the storage organ of the main medicinal parts and effective medicinal components, and the variety, content and proportion of sugar in the fruit are factors to determine the variety and its commercial value (Yao *et al*. 2011). When the environmental conditions are different, different soil and meteorological factors will have a certain impact on the active ingredients of wolfberry (Su *et al*. 2015; Abd El-Wahab *et al*. 2018). Therefore, the study of proteomics is helpful to study the differences of wolfberry in different habitats.

Conclusion

The proteins expression levels of the wolfberry were different between Ningxia and the other four habitats. The number of DEPs in Qinghai/Ningxia and Gansu/Ningxia was obviously more than that in Inner Mongolia/Ningxia and Xinjiang/Ningxia. And in the main functional items, the number of DEPs in Qinghai/Ningxia and Gansu/Ningxia was significantly higher than that in Inner Mongolia/Ningxia and Xinjiang/Ningxia. According to the above conclusions, it can be inferred that the wolfberry in Ningxia is significantly different from Qinghai and Gansu, and less different from Inner Mongolia and Xinjiang. This study may provide a reference for analyzing wolfberry quality in different habitats. However, the exact biological function and interaction among differentially expressed proteins need to be further studied.

Acknowledgement

We would like to thank the financial supports of National Natural Science Foundation (31660438) of China, Postgraduate Innovation Project of Ningxia University (2019), Innovation Platform Funds of Ningxia Key Laboratory for Food Microbial-Applications Technology and Safety Control (2018DPC05026), and Project of Support Local Colleges and University Reform and Development Funds of China (2018).

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