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Proteomic Profile of Edible Bird's Nest Proteins

Xiaoqing Liu,[†] Xintian Lai,[†] Shiwei Zhang,[†] Xiuli Huang,[§] Quanxue Lan,[†] Yun Li,[†] Bifang Li,[†] Wei Chen,[†] Qinlei Zhang,[†] Dezhi Hong,[#] and Guowu Yang*[,†]

[†]Shenzhen Academy of Metrology and Quality Inspection, Shenzhen 518102, People's Republic of China

[§]Huizhou Quality and Measuring Supervision Testing Institute, Huizhou 516003, People's Republic of China

[#]Shenzhen Pan Kai King Trade Company, Ltd., Shenzhen 518000, People's Republic of China

ABSTRACT: Edible bird's nest (EBN) is made of the swiftlets' saliva, which has attracted rather more attention owing to its nutritious and medical properties. Although protein constitutes the main composition and plays an important role in EBN, few studies have focused on the proteomic profile of EBN. The purpose of this study was to produce a proteomic map and clarify common EBN proteins. Liquid-phase isoelectric focusing (LIEF) was combined with two-dimensional electrophoresis (2-DE) for comprehensive analysis of EBN proteins. From 20 to 100 protein spots were detected on 2-DE maps of EBN samples from 15 different sources. The proteins were mainly distributed in four taxa (A, B, C, and D) according to their molecular mass. Taxa A and D both contained common proteins and proteins that may be considered another characteristic of EBN. Taxon A was identified using MALDI-TOF-TOF/MS and found to be homologous to acidic mammalian chitinase-like (*Meleagris gallopavo*), which is in glycosyl hydrolase family 18.

KEYWORDS: *edible bird's nest, protein, 2-DE, acidic mammalian chitinase, liquid-phase isoelectric focusing*

INTRODUCTION

Edible bird's nest (EBN) is made of the swiftlets' saliva secreted by salivary glue. EBN comes from four species of swiftlets (*Collocalia fuciphaga*, *Collocalia maxima*, *Collocalia germnis*, and *Collocalia unicolor*[1]) distributed in Malaysia, Thailand, Indonesia, and Vietnam. EBN has been proven to possess nourishing and medical properties. Studies have shown that the EBN extract can stimulate mitosis hormones to promote epidermal growth and inhibit infection of influenza viruses in a host range-independent manner.[2−4] Moreover, Matsukawa[5] found that oral administration of EBN extract improved bone strength and calcium concentration; in addition, dermal thickness was also increased in ovariectomized rats. Of these functions, protein is speculated to be the key factor, because protein is the major undertaker of life activities. Furthermore, protein is the major component of EBN, which consists of up to 60% of the mass of EBN.[6] Therefore, it is important to know the whole proteins and analyze in-depth the common proteins to unveil the secret of EBN.

Two-dimensional electrophoresis (2-DE) is the common method of choice for proteomic analysis.[7] Up to now, only two papers related to the research of EBN protein can be found. Qu et al.[8] found the major allergens in EBN were the 66 kDa proteins, which were found to be homologous to ovoinhibitor. However, only part of EBN proteins were included in their paper, because their aim was to find allergens in EBN and adopted an immunochemistry method. Wu et al.[9] used SYBR green PCR and 2-DE methods to authenticate edible bird's nest food. However, as the proteins were made merely by aqueous extraction and followed by using a ReadyPrep 2-D Cleanup kit, the proteins of EBN were not fully extracted. Moreover, some low-abundance proteins were not shown well; these two methods did not cover all of the proteins. Until now, no published papers have reported the protein profile of EBN and deeply explored the common proteins in EBN. Recently, we have reported the characteristic proteins of 106 and 128 kDa of EBN, which are high-abundance proteins,[10] but these two proteins could seriously influence the isoelectric focusing of 2-DE and conceal the low-abundance proteins. In this study, the proteins were divided into two parts (high-abundance proteins, which were reported in our previous study,[10] and low-abundance proteins) for preseparation and separation. Herein we concentrated on the low-abundance proteins research.

Liquid-phase isoelectric focusing (LIEF) was believed to be one of the most promising preseparation methods.[11] It separated proteins on the basis of their isoelectric points. With its high-enrichment capacity and low cross-contamination, many groups have utilized the LIEF-based strategy for studies.[12−15] In this paper, we adopted an ultrasonic treatment to extract proteins in a maximum amount; LIEF was used to reduce the complexity of EBN proteins and enrich relatively low-abundance proteins. 2-DE was followed to obtain the protein profile of EBN and analyze the diversity of proteins.[16] Proteomic maps of EBN were presented, and other characteristics of protein were detected.

MATERIALS AND METHODS

Materials and Apparatus. Rotofor cells used for LIEF, IPG strips, protein marker, and other related chemicals for electrophoresis were purchased from Bio-Rad (Hercules, California, USA). Double-distilled H_2O (ddH_2O) was used throughout the experiment. Uibracell VCX750 (Sonics and Materials, Inc., Newtown, CT, USA) was used for sample treatment. A PowerPac HV system and protein IEF (Bio-Rad) were used for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and 2-D PAGE analysis. An ABI 4800 MALDI-TOF-TOF/MS (ABI, Carlsbad, CA, USA) was used for MS analysis.

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EBN Sample Collection. Fifteen EBN samples were collected from Malaysia, Thailand, Indonesia, and Vietnam. Samples were labeled upon arrival at the laboratory and freeze-dried for 12 h. The nests were pounded in a mortar and then stored at −20 °C until protein extraction. The codes and descriptions of the samples were listed in Table 1.

Table 1. Information of EBN Samples

sample code	habitat	sample color	category
A011-1	Indonesia	white	house nest
A011-2	Indonesia	white	house nest
A012-1	Indonesia	white	house nest
A012-2	Indonesia	white	house nest
B011-1	Malaysia	white	house nest
B011-2	Malaysia	white	house nest
B012-1	Malaysia	white	house nest
B012-2	Malaysia	white	house nest
C011-1	Thailand	white	house nest
C011-2	Thailand	white	house nest
C011-3	Thailand	white	house nest
A021-1	Indonesia	white	cave nest
A021-2	Indonesia	yellow-white	cave nest
D021-1	Vietnam	white	cave nest
D021-2	Vietnam	white	cave nest

Crude Protein Extraction from EBN. Ground EBN samples (1.0 g) dissovled with 50 mL of ddH_2O were treated ultrasonically in an ice bath (pulse on, 2 s; pulse off, 4 s; ampl, 80%; 30 min) and centrifuged (10000 rpm for 10 min); the supernatants were dialyzed overnight against ddH_2O in a dialysis bag with a 3500 cutoff molecular weight; then the protein solutions were freeze-dried and stored at −20 °C until use.

Prefractionation by LIEF. Lyophilized protein was dissolved to 0.02 g/mL with 7 M urea, 50 mM DL-dithiothreitol (DTT), 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 1% ampholyte at a pH range of 3−10. The protein solution was loaded into the Rotofor cell according to the instructions of the manufacturer and run at 1 W constant power at 4 °C for 1.5 h. Electrolytes in the anode and the cathode champers were 0.1 M H_3PO_4 and 0.1 M NaOH. At equilibrium, the voltage were 600 V, and each fraction was collected to a 1.5 mL eppendorf tube and labeled 1−10 according to the pH arrangement from low to high. Fractions 1−3 were removed, and the rest of the fractions were collected for preseparation again; subsequently, fractions 5−9 fractions were collected to dialyze against 1000 mL of ddH_2O for 1.5 days at 4 °C with five changes. The desalted protein solutions were freeze-dried and stored at −20 °C until use. Five microliters of the crude proteins and Rotofor fractions were also analyzed by 10% SDS-PAGE, followed by Coomassie brilliant blue R-250 staining.

Two-Dimensional PAGE. The lyophilized protein prepared above was resolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, and 0.5% ampholyte), and the Lowry method used for protein quantitation. Each gel was loaded with approximately 130 μg of protein. The first-dimensional IEF was performed on 7 cm pH 4−7 linear IPG strips at 20 °C with a maximum current setting of 50 μA/strip using a Protean i12 IEF cell; the IEF procedure was as follows: (i) 100 V, step-and-hold, 1 h; (ii) 300 V, step-and-hold, 0:40 h; (iii) 500 V, step-and-hold, 0:40 h; (iv) 4000 V, gradient, 3 h; (v) 4000 V, step-and-hold, 02:30; (VI) 5000 V, gradient, 01:00;(VII) 5000 V, step-and-hold, 01:00. Two-dimensional SDS-PAGE assay was carried out according to the method of Zhu,[17] but with a concentration of 10%. The Precision Plus Protein (Bio-Rad) molecular weight standard was applied to each gel. Image analysis was processed by PDQuest 8.0.1 software (Bio-Rad).

MALDI-TOF-TOF/MS. Each individual protein spot was cut out and then digested in gel according to the method of Katayama.[18] Peptide mass fingerprint mapping was performed using an ABI 4800 MALDI-TOF-TOF/MS plus mass spectrometer. On the basis of combined MS and MS/MS spectra, proteins were successfully identified at 95% or higher confidence intervals of their scores in the Mascot v2.1 search engine (Matrix Science Ltd., London, UK), using the following search parameters: NCBInr-Animals; trypsin as the digestion enzyme; one missed cleavage site; fixed modifications of carbamidomethyl (C); partial modifications of oxidation (M) and acetyl (protein N-term); 50 ppm for precursor ion tolerance and 0.3 Da for fragment ion tolerance.

RESULTS AND DISCUSSION

Collection of EBN Samples and Extraction of Crude Protein. The collection of EBN samples was crucial to the present study. However, because there are many fake and low-quality EBN samples, the quality of EBN purchased from the general market cannot be guaranteed. With the help of Shenzhen Pan Kai King Trade Co., Ltd., 15 EBN samples were collected from 4 different countries of origin. These samples included 11 house nests (nests collected from inside free-standing human residences) and 4 cave nests (nests collected from caves).

EBN proteins were thoroughly dissolved in distilled water with ultrasonic treatment in an ice bath. Lyophilized crude proteins were weighed. We found the total concentration of crude protein to be similar to the value indicated by the Kjeldahl nitrogen method. This indicated that the protein extraction method was a suitable way to dissolve these EBN proteins.

Protein Preseparation. In a previous work, we studied highly abundant EBN proteins measuring 128 and 106 kDa and produced a 2-DE map of both.[10] In the present study, to evaluate low-abundance proteins and determine the functions of these proteins in EBN, the 128 and 106 kDa proteins were removed. Preseparation was necessary before the proteins could be separated by 2-DE. Good preseparation was found to be the key to good separation effects in 2-DE mapping.

Due to the complexity of the EBN protein profile, the low-abundance proteins were not well displayed on the 2-D gels using traditional precipitation methods such as trichloroacetic acid (TCA) precipitation, acetone precipitation, and TCA−ethanol precipitation. This was because all of the proteins were coprecipitated and the complexity of the sample could not be reduced or compartmentalized.

LIEF was used to address the variations in protein abundance across different samples. LIEF has been reported to separate and enrich low-abundance proteins derived from cell lysates, tissues, and plasma prior to MS analysis.[15] In this paper, LIEF was used to preseparate EBN proteins. On the basis of differences in isoelectric points, the compound was separated into 10 fractions. The pH of these fractions varied between 2.0 and 10.0. All 10 Rotofor fractions were subjected to 10% SDS-PAGE, which was used to analyze the effects of the first round of separation by LIEF. The 128 and 106 kDa proteins were mainly distributed in the first three fractions, especially fraction 1. To reduce the concentrations of these highly enriched proteins, 4−10 fractions were collected for separation with the Rotofor cell. As shown in Figure 1, after two rounds of preseparation, the 128 and 106 kDa proteins were still present in the first three fractions. Fraction 10 contained few proteins. These two fractions were removed. Proteins from fractions 5−8 were collected. This suggested that LIEF had successfully reduced the complexity of the EBN sample, that all of the EBN proteins were acidic, and that the IEP of these proteins ranged between 4 and 7 except for the 128 and 106 kDa proteins, for which IEPS were below 4.

As shown in Figure 2, after preseparation with LIEF, the low-abundance EBN proteins were enriched. Not many proteins were present in the EBN, and the proteins were mainly between 35 and 75 kDa in mass. The four protein bands produced by 15

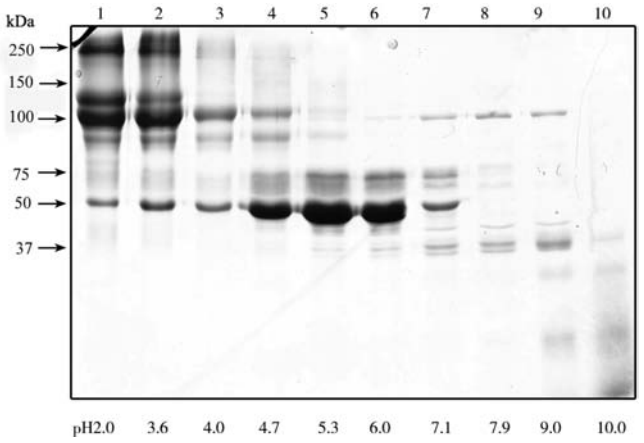

Figure 1. SDS-PAGE of 10 fractions separated by LIEF. A 5 μL aliquot of each Rotofor fraction was analyzed by 10% SDS-PAGE, and gels were stained with CBB-R250. The 128 and 106 kDa proteins were mainly distributed in one to three fractions, the lower abundance proteins were enriched in four to eight fractions.

EBN samples were named bands A, B, C, and D according to molecular mass.

EBN Proteome Maps and Identification of Proteins. To produce 2-DE maps of EBN, after preseparation with LIEF, 130 μg proteins were separated by 2-DE on 7 cm pH 4−7 IPG strips and stained with Coomassie brilliant blue R250. The 2-DE maps were produced in triplicate for each protein extraction experiment, and they showed a high level of reproducibility. Fifteen EBN protein maps are shown in Figure 3. These were analyzed using PDQuest software. About 20−100 protein spots were detected on the maps. The 2-DE pattern of EBN was relatively simple and the proteins were distributed in four taxa (A, B, C, and D) corresponding to the four bands (Figure 2). The proteins in each taxon had the same molecular weight and different isoelectric points. The proteins of taxon A were about 50 kDa in mass and were present in all EBN samples. They were highly abundant; four to five protein spots were attributed to this taxon. The proteins of taxon D were about 66 kDa in mass and present in all samples, although they were not obvious on the 2-DE map of A011-1, but they were faintly visible in band D of A011-1 (Figure 2); seven to nine protein spots were detected. The proteins of taxon B were about 40 kDa in mass and produced three to five protein spots. The proteins of taxon C were about 35 kDa in mass and produced three protein spots. The abundances of taxa B and C differed across each EBN sample. Because of the similar molecular weights and low abundance, the protein spots of taxa B and C existed in mixed form in most EBN samples, except for A011-1, A011-2, B012-1, and C011-3, in which they were clearly distinguishable.

Taxa B and C may represent the diversity of proteins, but the existence of these taxa did not correspond with the place of origin and classification of EBN. We considered the diet of swiftlets to be the cause of the diversity among taxon B, taxon C, and also other small proteins. This view was generally in agreement with findings reported by Lourie.[19] Although there were several ordered spots (including those of taxa A and D), each taxon tended to present an isoform that underwent post-translational modifications. Glycosylation and phosphorylation were found to influence the isoelectric points. This phenomenon is widespread in human and swine saliva.[20,21] Qu found that EBN allergens have the same N-terminal sequence and glycan chains.[8] For this reason, we believe that post-translational modifications may be widespread in EBN proteins. We found that taxa A and D existed stably and were common in all EBN samples. They may be representative of EBN proteins and may even be another characteristic of EBN. (The first characteristic proteins of EBN are described in our previous study.[10]) The establishment of the EBN proteome profile was found to be important for the medical effects of each protein.

Mass spectrometric analysis here facilitated a more affirmative confirmation of the identities of the common EBN proteins. In the 2-DE map, the existence of taxon D was found to be consistent with findings reported by Qu.[8] Taxon D was considered an EBN allergen homologous to ovoinhibitor, a Kazal type serine protease inhibitor found in egg whites. We suggest that children with histories of hypersensitivity should not eat ENB. Mass spectrometric analysis was also carried out on the in-gel digested tryptic peptides of each protein spot in taxon A. The peptide mass fingerprint results derived from MALDI-TOF-TOF/MS analysis were used to search against the Mascot v2.1 search engine and NCBI nonredundant databases. All of the proteins in taxon A were found to be homologous acidic mammalian chitinase-like fragments (AMCase-like) originating from *Meleagris gallopavo*. This phenomenon is probably attributable to the fact that the swiftlet genome is not completely sequenced and to the partial sequence homology of *M. gallopavo* proteins. The identified protein ID was gi|326933899|ref|XP_003213035.1, the theoretical isoelectric point was 9.53, and the coverage rate was >36.2%. Here, only protein spot 3 on

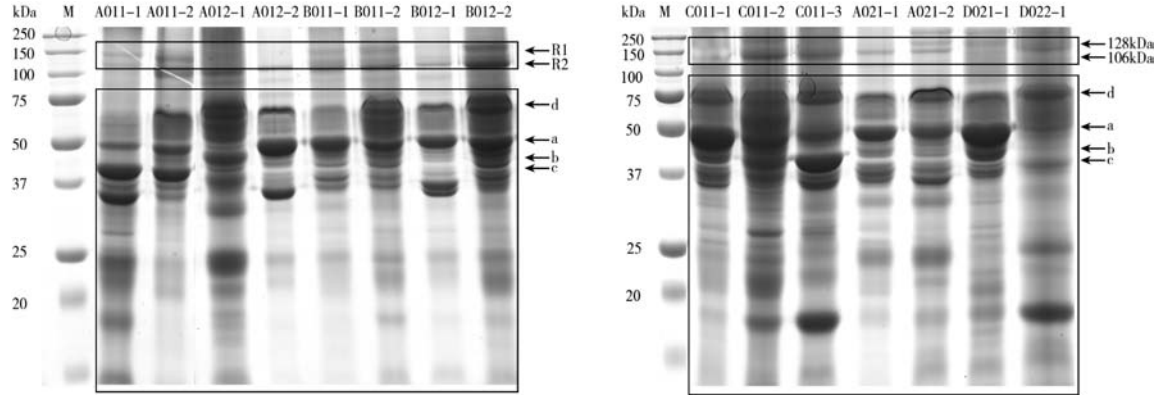

Figure 2. SDS-PAGE of low-abundance proteins of 15 EBNs separated by LIEF. Four protein bands of the samples were named bands A−D on the basis of the molecular size.

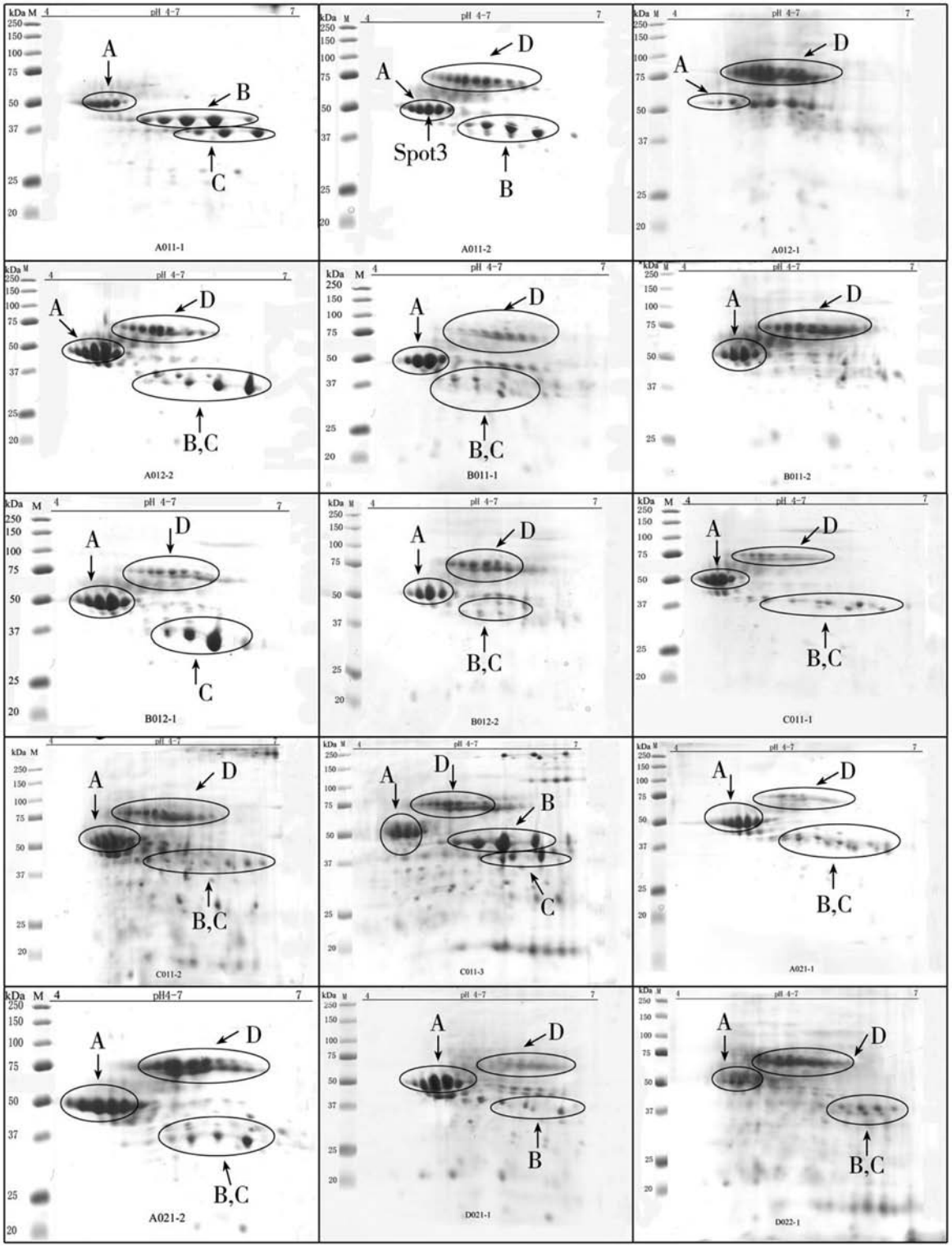

Figure 3. 2-DE maps of 15 EBN samples. After preseparation by LIEF, the 15 EBN protein samples were separated by 2-DE on 7 cm, pH 4−7 IPG strips and then run on 10% SDS-PAGE gels. M indicates the molecular mass standards. Compared with 2-DE maps, the proteins distributed in four taxa (A, B, C, D), which correspond to the four bands (Figure2). We found that taxa A and D exhibited stability and were common in all EBN samples; they can be other characteristic proteins of EBN.

the map of A011-2 in Figure 3 is listed in Table 2. AMCase-like (*M. gallopavo*) contained 221 amino acid residues and had a theoretical molecular mass of 24733.2 Da. However, the actual value (50 kDa) was double that of the theoretical value. This is consistent with our view of the proteins of taxon A as dimers, and there may exist some chemical bonds that SDS and urea cannot break (all SDS-PAGE experiments were run under reducing conditions). The isoelectric point was different from the actual value (p*I* 5−6), and the different protein spots in taxon A were attributed to the same AMCase-like fragment. We attributed this

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Table 2. Homologous Peptide Sequences of Protein Spot 3 Identified by MALDI-TOF-TOF/MS[a]

MGGNAPHFCR	**SR**SRAARPRG	FMAPPRGAAR	RSTQPSPALP	PPGGNPQSTA
YVLSCYFTNW	AQYRPGLGKY	MPDNIDPCLC	DHLIYAFAGM	SNNEITTYEW
NDETLYK**SFN**	**GLKNQNGNLK**	**TLLAIGGWNF**	**GTAK**FSTMVS	TPENRQTFIK
SVIK**FLRQYQ**	**FDGLDIDWEY**	**PGSR**GSSSQD	KGLFTVLVQE	MLAAFEQEAK
QVNKPRLMIT	**AAVAAGLSNI**	**Q**		

[a]Boldface type indicates matching peptides. Spot 3 was on the map of Figure 3 A011-2.

to the presence of the post-translational modifications. Chitinases are ubiquitous chitin-fragmenting hydrolases. AMCase belongs to glycosyl hydrolase family 18.[22] It has a C-terminal chitin-binding domain and a 39 kDa catalytic domain with a TIM-barrel structure. It often exists in mammals and has been most often studied in humans and rodents.[23,24] However, it was first discovered in EBN. The existence of AMCase-like fragments may help the esculent swift to resist chitin biological invasion, but whether it still possesses enzymatic activity in EBN remains an issue. We will purify this protein for further study.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-0755-29561753. Fax: +86-0755-27528854. E-mail: Yangguowu@yahoo.com.cn.

Notes

The authors declare no competing financial interest.

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