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Original research article

A comprehensive proteomics study on edible bird's nest using new monoclonal antibody approach and application in quality control

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ABSTRACT

Edible bird's nest (EBN) is a glue-like substance deriving from salivary secretion by specific swiftlets, and protein is considered as the main component of EBN. Accounting over 50% by weight, the exact identities of EBN proteins are still not well understood, due to difficulties of extraction, purification and identification. By using EBN proteins as antigens, 31 monoclonal antibodies specifically against the proteins were generated. The proteins of EBN were subjected to identification by shotgun proteomics. Six protein identities were revealed, including acidic mammalian chitinase (AMCase)-like, mucin 5AC-like and ovoinhibitor-like proteins. In parallel, the monoclonal antibodies were used to immunoprecipitate proteins from EBN extract, and subsequently the precipitated product(s) was identified. AMCase-like protein was most frequently precipitated by the antibodies. The existence of AMCase-like protein in EBN was further verified by: (i) recognition of chicken AMCase by our anti-EBN antibodies; and (ii) recognition of EBN AMCase-like protein by a commercial anti-AMCase antibody. The antibody was highly sensitive and selective to AMCase-like protein in EBN products, with limit of detection at 0.01 μg/mL in ELISA test. Thus, AMCase-like protein, or its antibody, could be used as a new quality control marker for EBN.

1. Introduction

Edible bird's nest (EBN; cubilose) is the salivary secretion from specific swiftlets, e.g. *Aerodramus fuciphagus* and *Aerodramus maximus* (Kang et al., 1991). Because of its high protein content, EBN is being used as a health food supplement commonly in Asia. *Aerodramus* family is mostly distributed in South East Asia, and Indonesia, Malaysia, Vietnam and Thailand are the major producers of EBN. Swiftlets can produce EBN as the "house" for their fledglings during the breeding season, and they leave the nest when young birds are normally 3 months old. Farmers then collect the empty EBN in swiftlet houses or caves. After labor-intensive cleaning processes, EBN is exported to China, Hong Kong, Singapore, US and Canada. Owing to limited supply and labor intensive processing, EBN often fetches a high price with a range from USD 1,000–15,000 per kilogram and is known as the "caviar" of the East (Marcone, 2005).

Because of its remarkable health benefits and medicinal functions, EBN has been served as an esteemed food since the Tang dynasty (A.D. 675) in China (Koon and Cranbrook, 2002). Today, EBN is proposed to possess various functions, including anti-inflammatory, anti-influenza, anti-oxidant, skin whitening, bone strength improvement and epidermal growth enhancement (Kong et al., 1987, 1989; Guo et al., 2006; Aswir and Wan Nazaimoon, 2011; Matsukawa et al., 2011; Yew et al., 2014; Chan et al., 2015). Protein is a major part of EBN, averaging around 50% of dried weight of EBN (Jiangsu New Medicine College (Former of Nanjing Medical University), 1977; Wong et al., 2017); it is conjectured to be an important factor of the nutritional and medicinal functions of EBN.

However, proteomics research of EBN has reached a bottleneck, due to its poor solubility and low extractive rate. The proteins of EBN are often extracted with distilled water heated to 60–100 °C. No pure protein has been obtained despite different kinds of methods having been employed, such as centrifugation, electrophoresis, gel filtration, high performance liquid chromatography (HPLC) and ammonium sulfate precipitation (Lin et al., 2006). Streaking around the protein bands is commonly observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This circumstance probably is due to the unusual richness of high density glycoproteins within EBN (Marcone,

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2005). The lack of a full *Aerodramus* genome sequence is another critical obstacle for proper protein identification.

To tackle this issue, a method was designed using monoclonal antibodies raised by hybridoma technology in this study. Monoclonal antibodies are promising tools in protein recognition, binding to the same epitope with a high specificity. Instead of traditionally starting with ultra-purified protein/peptide, a mixture of uncharacterized protein entities from EBN was applied to a number of hosts to provoke multiple immune responses. By using EBN soluble proteins as antigens, thirty-one monoclonal antibodies were produced by hybridoma technology: they were used here to fish out specific EBN proteins. After immunoprecipitation followed by LC–MS/MS analysis, we searched the protein sequence from the library of *Chaetura pelagica*, a bird species within the same family as *A. fuciphagus*. Specific proteins recognized by monoclonal antibodies were identified and validated. These monoclonal antibodies against EBN proteins could be used as tools in quality surveillance of EBN in commercial markets.

2. Materials and methods

2.1. Materials and sample collection

Ten batches of EBN cup from Indonesia, Malaysia, Thailand and Vietnam were randomly purchased in a Hong Kong market. The samples were labeled and stored at room temperature upon arrival in the laboratory. The sample information is listed in Table S1, and the morphology of EBN samples is shown in Fig. 1. The fake products of EBN, including agar, white fungus, gelatin, fried porcine skin and carrageenan were bought from local markets. 3,3′,5,5;-Tetramethylbenzidine (TMB) and peroxidase (HRP)-conjugated anti-mouse secondary antibody were purchased from Thermo Fisher Scientific (Waltham, MA). Anti-AMCase antibody was ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G agarose was obtained from Roche (Basel, Switzerland), Ultrapure water was processed by Millipore Milli-Q Advantage A10 system with 18.2 MΩ·cm (Billerica, MA). Protein assay dye was purchased from Bio-Rad (Hercules, CA), LC–MS grade acetonitrile and water were obtained from JT Baker (Center Valley, PA).

2.2. Protein extraction of EBN

An EBN cup, normally 5–10 g, was accurately weighed and soaked in 100 folds of water (*w/v*) for 3–15 h (3 h for White EBN; 10 h for Yellow EBN; 15 h for Red EBN), as reported previously (Chan et al., 2013b). After removing the soaking water, the EBN was washed with 500 folds of water (w/v) three times. The soaked EBN was put into a ceramic stewing pot with 30 folds of water and stewed for 8–40 h at 100 °C until it was totally molten (8 h for White EBN; 16 h for Yellow; 40 h for Red EBN). The sample was dialyzed overnight against water in a dialysis bag with 2000 molecular weight cut-off and then filtered, freeze-dried and stored at 4 °C until use. The protein extraction rate of EBN was quantified by Bradford protein assay, and the absorbance was measured at 595 nm.

2.3. Production of EBN monoclonal antibodies

White EBN (sample 1) extract was prepared by the method described in Section 2.2. The white EBN extract monoclonal antibodies were produced via hybridoma technology (Tsim et al., 1988). Firstly, the EBN extract was injected into 6 mice. The B cells from the spleen were isolated and cultivated with myeloma cells. After fusion of myeloma and B cells, all cell lines from 6 mice were separated and prescreened. Only thirty-one clones with high affinity were selected and the monoclonal antibodies were separated accordingly.

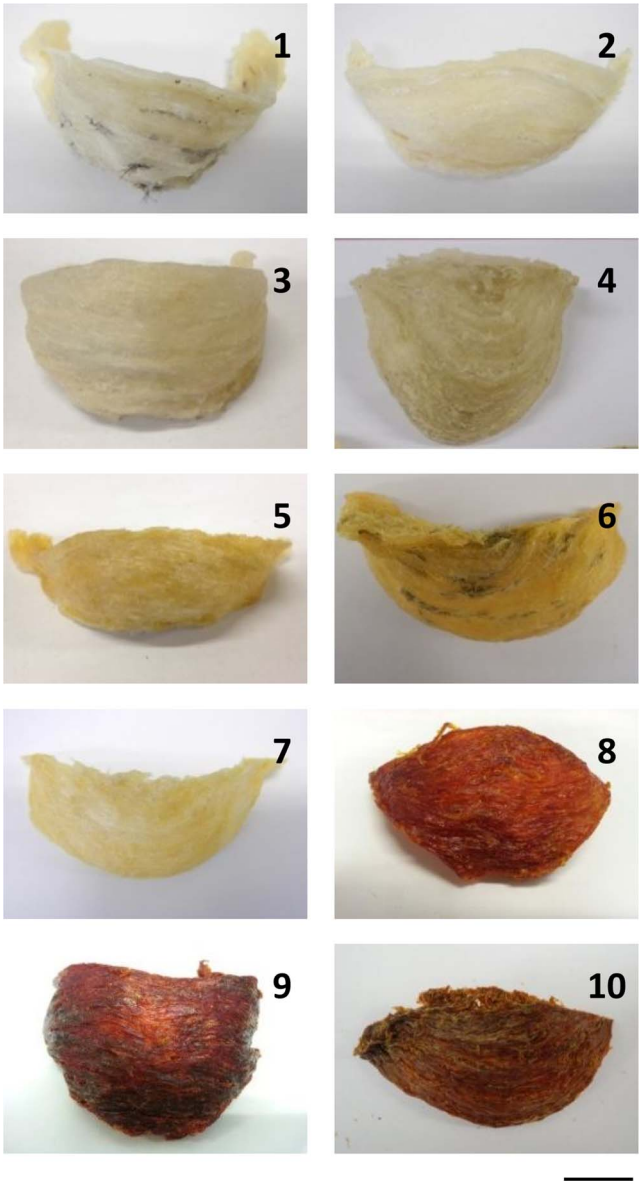

Fig. 1. The morphology of Edible bird's nest (EBN) samples.
The morphology of EBN (samples 1–10) used in this study. Bar: 1 cm.

2.4. Immunoprecipitation

Ten mg EBN powder were dissolved in 1 mL water, and 200 µL Protein G agarose were washed with 1 mL water twice, according to the manual. A pre-cleaning step was performed by adding 20 µL Protein G agarose to the solution; and rotated at 4 °C for 1 h. The solution was centrifuged at 3000 rcf for 3 min, and the supernatant was transferred to a new tube. A 20 µL aliquot of monoclonal antibody solutions was added to the solution and rotated at 4 °C for 18 h. Then, 30 µL Protein G agarose was added to the solution and rotated for another 4 h at 4 °C. The solution was washed twice with low salt lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 1 mM EDTA and 1 mM EGTA). The pellet was dissolved in water and heated at 95 °C for 5 min. The supernatant was subjected to LC–MS/MS for protein identification.

2.5. Protein identification by LC–MS/MS

After immunoprecipitation, the resulting proteins were then digested by trypsin (1:50 w/w; Promega, Madison, WI) overnight at 37 °C. The digested samples were desalted by C18 reverse-phase ZipTip