EXTRACTION AND IDENTIFICATION OF PROTEINS FROM EDIBLE BIRD'S NEST

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Specially dedicated to my beloved mother, father, and families.
And to my supervisors, Assoc. Prof. Dr. Chua Lee Suan, Dr. Zaidah and caring friends for endless help and support.

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ABSTRACT

Edible bird's nest (EBN) is a delicacy rich in proteins and carbohydrates from the salivary secretion of swiftlets. There are limited studies on the protein profile of EBN, mainly due to its complexity in chemical composition and diversity of bird species, as well as the limitation of analytical techniques. Therefore, in the current study, a number of protein extraction methods, including water sonication, Triton X-100 (non-ionic) and sodium dodecyl sulfate (SDS, ionic) detergent-assisted methods, and Tris-HCl buffer solubilization were used to compare the protein profiles of EBN harvested from Batu Pahat and Kota Tinggi in Malaysia. The yields of protein extracted from the EBN samples were determined by using Bradford assay. The water sonication and Triton X-100 extraction methods produced higher protein content (6.44-12.88 mg/g) than the SDS assisted and Tris-HCl buffer extraction methods (3.47-8.60 mg/g). Based on gel electrophoresis, EBN from Batu Pahat (17-150 kDa) exhibited more protein bands than those samples from Kota Tinggi (25-154 kDa). The difference could be explained by the difference in environment and food sources of swiftlets. Additional protein bands (25, 27 and 92 kDa) which were observed in the detergent-assisted methods were suggested to be either membrane or transmembrane proteins. After trypsin digestion, the presence of proteins was analyzed by liquid chromatography coupled with tandem mass spectrometry. The mass spectra revealed that acidic mammalian chitinase was the most abundant protein. The newly found proteins include pre-rRNA-processing protein TSR1 homolog isoform X3, collagen alpha-1(VII) chain-like, lysyl oxidase homolog 3 and phospholipase A2-like. As a summary, the protein extraction methods used in this study could produce good quality of proteins for affirmative confirmation using gel electrophoresis and mass spectrometric identification.

ABSTRAK

Sarang burung boleh dimakan (EBN) adalah makanan yang kaya dengan protein dan karbohidrat dari rembesan air liur burung layang-layang. Kajian saintifik terhadap profil protein EBN adalah agak terhad, disebabkan kerumitan komposisi kimia dan kepelbagaian spesis burung, serta keupayaan teknik analisis yang terhad. Oleh itu, dalam kajian ini, kaedah pengekstrakan protein termasuk sonikasi air, bantuan detergen Triton X-100 (bukan ionik) dan natrium dodekil sulfat (SDS, ionik), dan pelarutan dalam penimbal Tris-HCl telah digunakan untuk membandingkan profil protein EBN yang dituai dari Batu Pahat dan Kota Tinggi di Malaysia. Hasil protein yang diekstrak dari sampel EBN telah ditentukan dengan menggunakan cerakin Bradford. Kaedah sonikasi air dan Triton X-100 menghasilkan kandungan protein yang lebih tinggi (6.44-12.88 mg/g) daripada kaedah pengekstrakan bantuan SDS dan penimbal Tris-HCl (3.47-8.60 mg/g). Berdasarkan elektroforesis gel, EBN dari Batu Pahat (17-150 kDa) mempamerkan lebih jalur protein berbanding sampel dari Kota Tinggi (25-154 kDa). Perbezaannya boleh dijelaskan oleh perbezaan persekitaran dan perbezaan sumber makanan burung layang-layang. Jalur protein tambahan (25, 27 dan 92 kDa) yang diperhatikan dalam kaedah bantuan detergen dicadangkan sama ada protein membran atau protein transmembran. Selepas penghadaman dengan tripsin, kehadiran protein dianalisis dengan kromatografi cecair bergandingan spektrometri jisim. Spektrum jisim mendedahkan bahawa kitinase mamalia berasid merupakan protein yang paling banyak. Protein baharu lain yang diperoleh termasuk pra-rRNA-pemprosesan protein TSR1 homolog isoform X3, kolagen alpha-1(VII) berantai, homolog oksidase lisil 3 dan fosfolipase A2. Ringkasnya, kaedah pengekstrakan protein digunakan dalam kajian ini boleh menghasilkan kualiti protein yang baik untuk pengesahan afirmatif dengan menggunakan elektroforesis gel dan identifikasi spektrometrik jisim.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

μg	-	Microgram
μl	-	Microliter
2-DE	-	Two dimensional electrophoresis
AMC	-	Acidic mammalian chitinase
APS	-	Ammonium persulfate
BCA	-	Bicinchoninic acid
BSA	-	Bovine serum albumin
cm	-	Centimeter
CMC	-	Critical micelle concentration
DTT	-	Dithiothreitol
EBN	-	Edible bird's nest
EGF	-	Epidermal growth factor
g	-	Gram
hADSCs	-	Human adipose-derived stem cells
HPLC	-	High Performance Liquid Chromatography
IAA	-	Iodoacetamide
kDa	-	Kilo Dalton
LC-MS/MS	-	Liquid Chromatography – Tandem Mass Spectrometry
mg	-	Milligram
ml	-	Mililiter
mM	-	Millimolar
MS	-	Mass spectrometry
MWCO	-	Molecular weight cut off
NANA	-	N-acetylneuraminic acid
NCBI	-	National Centre for Biotechnology Information

NKEA	-	National Key Economic Area
ppm	-	Part per million
rpm	-	Rotation per minute
SA	-	Sialic acid
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCM	-	Traditional Chinese Medicine
TEMED	-	N,N,N,',N'-tetramethylethylenediamine
TNF-α	-	Tumour necrosis factor-alpha

LIST OF SYMBOLS

%	-	Percentage
V	-	Volt
°	-	Degree
$	-	United State Dollar
<	-	Less than
±	-	Plus minus
-	-	Minus

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Research background

Research into development of bioactive peptides from food based materials has increased steadily in recent decades. Indeed, food proteins play a significant role in improving human health beyond their nutritional value (Hartmann and Miesel, 2007). Therefore, foods that are rich in protein content are in high market demand, especially for consumers who are looking for healthy lifestyle (Bogdanov, 2011). Among the food based materials, edible bird's nest (EBN) has been the prime choice of natural food source for many consumers, particularly from Eastern countries.

EBN is the natural food source secreted from the two sublingual glands of *Aerodramus* genus, or commonly known as swiftlet or 'burung wallet' in Malaysia (Marcone, 2005). It is recognized as a delicacy, medicine and an important agricultural product. EBN is an important Chinese cuisine in ancient China. The consumption of EBN can be traced back, as early as the Tang dynasty in AD 618 (Marcone, 2005). To date, the consumption of EBN is believed by the Chinese community to relieve the problem of phlegm, gastric trouble, renal functions, libido, asthma, cough, tuberculosis, as well as to strengthen immune system, the growth of children, energy and metabolism (Hobbs, 2004). Numerous studies reported the

diverse biological activities of EBN. The earliest scientific evidences included the presence of epidermal growth factor (EGF)-like activity (Kong *et al*., 1987), anti-influenza virus and haemagglutination-inhibitory activities (Guo *et al*., 2006), anti-inflammatory effect (Vimala *et al*., 2011) and improvement of bone strength and dermal thickness (Matsukawa *et al*., 2011). To the best of our knowledge, the role of EBN in those biochemical mechanisms either *in vivo* or *in vitro* is still remaining unknown. Protein is speculated to be the key factor contributing to the biological activities (Chua *et al*., 2014). Furthermore, protein is the major component in EBN which covers for about 60% of the total mass, followed by 30% of carbohydrate (Chua *et al*., 2014). Therefore, it is not surprising that protein and carbohydrate could be the bioactive markers for the biological activities. The technical information about the protein profile and protein characteristics is essential to unveil the biological activities and nutritional value of EBN.

There are limited studies focusing on EBN protein based on the literature survey. Wu *et al*. (2010) used SYBR green ($C_{32}H_{37}N_4S^+$) polymerase chain reaction (SYBR green PCR) and two-dimensional electrophoresis (2-DE) methods to authenticate EBN based foods. However, the whole proteome of EBN might not be extracted by aqueous extraction and followed by a ReadyPrep 2-D Cleanup kit. In particular, some of the low abundance proteins could not be extracted. A study conducted by Ou *et al*. (2001) who found the major allergen (66 kDa) in EBN, which was reported to be homologous to ovoinhibitor. This Kazal-type serine protease inhibitor is one of the dominant allergens in chicken white egg. Besides, the detection of bioactive peptide (50 kDa) reported to be homologous acidic mammalian chitinase –like fragments (AMCase-like) originating from *Meleagris gallopavo* (Liu *et al*., 2012). It was first discovered in EBN and the existence of this fragment may help the esculent swift to resist chitin biological invasion. Nevertheless, the enzymatic activity of this fragment remains unclear. This finding has created more intensive research on protein or peptide in EBN recently. EBN still contains many proteins to be discovered, particularly for pharmacological applications.

1.2 Problem Statement

Protein extraction is challenging and result inconsistency. This technical problem has long been an issue for scientists (Mehmeti *et al*., 2011). Finding the best method of cell lysis for protein extraction is the preliminary step in the detection and identification of proteins in proteomics. To our knowledge, there is no single universal protein extraction method that can obtain all types of proteins, mainly because of the diversified properties of proteins. The protein extraction method varies significantly depending on what kind of sample matrix, the relative abundance of protein of interest, as well as the presence of high abundance protein that may obscure dissolution, detection and subsequently analysis of lower abundance protein (Ivanov and Lazarev, 2012). The ideal protocol must be highly reproducible with the minimal artifactual protein degradation for both gel electrophoresis and liquid chromatography mass spectrometry analysis (Kota and Goshe, 2011). Many techniques including mechanical and chemical methods are available for cell disruption and protein extraction. These techniques have been used by many researchers in their works for different purposes (Grabskia, 2009). However, there is still lacking of standard protein extraction procedures as a bottleneck to further studies on protein content in EBN. Sample preparation is a vital step in a gel-based proteomics approach and is absolutely essential for reproducible results (Rampitsch *et al*., 2006). Therefore, four different extraction methods, namely water, Triton X-100 (non-ionic), SDS (ionic) detergent lysis and Tris-HCl (pH7.5) buffer lysis were applied in this study to compare the methods that can give the highest number of protein and protein quality.

Recent technical advancement in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS) have made the effort of protein separation and identification to be faster and reliable in accuracy and sensitivity. There are limited studies on proteins from EBN samples from gel electrophoresis up to LC-MS/MS. Several methods have been reported for protein extraction from EBN. Most studies on EBN protein were carried out just up to the stage of protein size determination using SDS-PAGE without protein identification.

This might due to the objective of the researchers who only would like to know the electrophoretic fingerprinting for EBN. Therefore, this study was further studied up to the protein identification based on mass spectrometric approach.

To the best of our knowledge, there are also very few studies which focused on protein from EBN. Mostly, studies have been concentrated on the proximate analysis of crude protein from EBN (Marcone, 2005; Nurul Huda *et al*., 2008; Zainab *et al*., 2013 and Wong *et al*., 2014). In Malaysia, many studies have been carried out on EBN for their biological activities such as antioxidant (Engku Hanisah *et al*., 2014; Yida *et al*., 2014 and Elicia *et al*., 2014), anti-inflammatory effect (Vimala *et al*., 2011), chondro-protective agent (Chua *et al*., 2013) and many more. But the specific compounds which attributes the biological properties is not fully understood. In particular, the study of EBN protein identification from Malaysia is very limited. Therefore, the kind of EBN protein presents in Malaysian EBN is still an unknown. Although several studies have been carried out by foreign researchers, especially from China, Hong Kong and Singapore, the composition of EBN protein varies according to their geographical origins (Norhayati *et al*., 2010; Liu *et al*., 2012). This could be due to the difference in food and metabolism which might affect the protein characteristics of swiftlets (Zainab *et al*., 2013). Thus, the protein content of Malaysian EBN shall be conducted in detail.

1.3 Objectives

The main objective of this research was to identify proteins from EBN samples.

1.4 Scope of Study

To achieve the above mentioned objective, the following scopes of study are designed:

i. To extract proteins from EBN samples using different extraction methods, namely water, Triton X-100 (non-ionic) detergent lysis, SDS (ionic) detergent lysis and Tris-HCl (pH7.5) buffer lysis.

ii. To determine protein quality and size based on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

iii. To identify the proteins of EBN samples using peptide mass fingerprinting matching to Uniprot protein database.

1.5 Significance of the Study

EBN has been broadly consumed around the world and its beneficial effects are enormous. With the increasing awareness among EBN consumers, the technical information on the nutritional value of EBN and the pharmacological activities are of importance for further product formulation and development with promised quality. Through this study, the scientific data can be used to increase the value of EBN, as well as to increase the confidence of consumers on EBN products. This will promote EBN production and profit earning for our country.

Most of the factual findings for EBN in this study was gathered from views, proposals, experiences, visions and information contributed by various experts in the bird-nest industry. Based on literature survey, the therapeutic effects of EBN have

been proven by local community (Lim *et al*., 2002). Because of the proven biological effects, the phenomenon EBN adulteration is getting serious nowadays, especially under the scenario of limited supply of natural EBN. The wide array of EBN varieties has created the need to authenticate. Thus, the protein profile of EBN could be used to authenticate EBN with high confident level. Moreover, the protein information from this study will be useful for the explanation of biological activity from EBN. The protein identification will also provide a preliminary data for future nutraceutical and cosmeceutical research. The conventional supplements which are chemically synthesized might pose threat to human health, and the chemical used could be accumulated in human body over long period of medication. Therefore, the demand for natural supplements is preferable by consumers. EBN based natural products will definitely become another alternative for the chemically synthesized supplements.

A high throughput approach of LC-MS/MS for EBN protein analysis was applied in this study. LC-MS/MS combined the advantage of liquid chromatographic separation and mass spectrometric detection, thus providing a higher selectivity and accuracy in data interpretation. Previously, the application of LC-MS/MS is mostly for the small metabolites, but the profiling of biomolecules, particularly protein in EBN samples is very limited. The integration of protein extraction method and LC-MS/MS can determine and identify protein in EBN with high accuracy.

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