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PHYSICAL CHARACTERISTICS, MORPHOLOGY AND PROTEIN PROFILES OF SALIVARY GLANDS OF TWO LOCAL EDIBLE NEST SWIFTLET SPECIES

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By

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In the age of twenty centuries, edible bird nest (EBN) was highly contributed towards most of the Asian countries economy. However, as the EBN is constructed from saliva of the swiftlet, association between the nest and the salivary glands is still obscure. This is a fundamental study providing valuable information about the salivary glands of EBN swiftlets. The first objective of this study was to compare the morphological characteristics of Malaysia's EBN swiftlets and examination of the histomorphology of the salivary glands. The second objective which is the main part of the study was to profile and identify the difference in protein spots from the salivary glands of EBN swiftlets. The last objective was to profile the glycoproteins of EBN swiftlets salivary gland using lectin based detection method. A total of 20 birds were caught in the Gomantong Cave, Sabah, out of which, 10 of it were *A. fuciphagus* and the balance were *A. maximus*. Both species were compared morphologically and it shows that *A. maximus* are larger in body size compared to *A. fuciphagus*. The sublingual gland on the other hand shows that, both species have morphologically similar including the size. The tissue was then stained with H&E stain and results show that both species generally have quite similar tissue structure. Combination of Alcian Blue-Periodic Acid Schiff shows both species are rich in mixture of acidic and neutral mucins secretion. However, *A. fuciphagus* has greater neutral mucin secretion compared in *A. maximus*. Based on relative sublingual glands weight, comparison between female *A. fuciphagus* and female *A, maximus* are the only one that shows significantly difference. Proteomics studies commenced with the protein quantification and *A. fuciphagus* male has the highest concentration of protein which is 8.675 µg/µL followed by *A. fuciphagus* female 6.875 µg/µL, while in male and female *A. maximus* consist of 5.743 and 6.117 µg/µL of proteins respectively. 2D gel was performed to obtain the salivary gland protein profiles and

the differential proteins expressed were identified using LC/MS Q-ToF mass spectrometry analysis. Profiling of the glycoproteins shows most of the glycoproteins present are the N-link glycoproteins and conjugated with sialic acid.

Keywords: Edible bird nest (EBN), swiftlets, salivary glands, protein, glycoprotein

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

FIZIKAL KARAKTERISTIK, MORFOLOGI DAN PROFIL PROTEIN DARI KELENJAR AIR LIUR DARIPADA DUA SPESIS BURUNG WALIT 'EBN' TEMPATAN

Oleh

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Dalam abad ke-20 ini, sarang burung yang boleh dimakan (*edible bird nest, EBN*) sangat menyumbang kepada sebahagian besar ekonomi negara-negara Asia. Walaubagaimanapun, seperti yang diketahui bahawa EBN dihasilkan dari air liur, akan tetapi kaitan di antara sarang dan kelenjar masih tidak jelas. Kajian ini merupakan kajian asas yang akan menghasilkan lebih pelbagai maklumat tentang kelenjar air liur burung walit *EBN*. Objektif pertama kajian ini adalah untuk membandingkan morfologi ciri-ciri morfologi burung walit EBN Malaysia dan pemeriksaan histomorphology kelenjar air liur. Objektif kedua yang merupakan bahagian utama kajian ini adalah untuk mengenal pasti profil dan perbezaan protein yang terdapat dalam kelenjar air liur burung walit *EBN*. Manakala objektif terakhir adalah untuk memprofilkan glikoprotein dalam kelenjar air liur burung walit *EBN* menggunakan pengesanan berasaskan lektin. Sebanyak 20 ekor burung telah ditangkap di Gua Gomantong, Sabah, dimana 10 daripadanya adalah *A. fuciphagus* dan selebihnya adalah *A. maximus*. Kedua-dua spesis telah dibandingkan dari segi morfologi dan hasilnya menunjukkan *A. maximus* mempunyai saiz badan yang lebih besar berbanding *A. fuciphagus*. Kalenjar sublingual pula menunjukkan bahawa, kedua-dua spesis mempunyai morfologi yang sama termasuk saiznya. Tisu itu kemudian diwarnakan dengan H&E warna dan keputusan menunjukkan bahawa kedua-dua spesies umumnya mempunyai struktur tisu yang hampir sama. Gabungan warna Alcian Blue-Berkala Acid Schiff menunjukkan kedua-dua spesies kaya dengan campuran rembesan berasid dan mucins neutral. Walaubagaimanapun, *A. fuciphagus* mempunyai rembesan mucin neutral yang lebih tinggi berbanding *A. maximus*. Berdasarkan berat relatif kelenjar sublingual, hanya perbandingan antara betina *A. fuciphagus* dan betina *A. maximus* yang menunjukkan perbezaan ketara. Kajian proteomik dimulakan dengan kuantifikasi protein dan *A. fuciphagus* jantan mempunyai kepekatan protein tertinggi iaitu 8.675 μg/uL diikuti oleh *A. fuciphagus* betina 6.875 μg/uL, manakala jantan dan betina *A. maximus* masing-masing mempunyai kepekatan protein pada 5.743 dan 6.117 μg/uL. Gel 2-dimensi (2D)

dilakukan untuk membentangkan profil protein dari kelenjar air liur dan seterusnya, protein yang menunjukkan perbezaan di antara mereka di kenal pasti menggunakan kaedah analisis spektrometer jisim LC/MS Q-ToF. Profil glikoprotein yang dijana menggunakan kaedah pengesanan berasaskan lektin menunjukkan kebanyakan glikoprotein yang dijumpai adalah glikoprotein N-link dan glikoprotein yang dikonjukasi dengan asid sialik.

Kata kunci: Sarang burung yang boleh dimakan (EBN), burung walit, kalenjar air liur, protein, glikoprotein

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This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

%	Percent
µm	Micrometre
mm	Millimetre
cm	Centimetre
µM	Micromolar
ng	Nanogram
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
sec	Second
min	Minute
hr	Hour
V	Voltage
kVh	Kilovolt per hour
W	Watt
mA	Milliampere
Da	Dalton
kDa	Kilo Dalton
µL	Microliter
mL	Mililitre
L	Litre
mM	Milimolar
M	Molar
nm	Nanometre
^{o}C	Degree Celsius
pm	Post meridiem
xg	Times gravity
m/z	Mass-to-charge ratio
rpm	Rotations per minute
3D	Three dimension
AF	*Aerodramus fuciphagus*
AM	*Aerodramus maximus*
EBN	Edible bird nest
H&E	Hematoxylin and Eosin
AB	Alcian blue
PAS	Periodic acid Schiff
X	Times
SD	Standard deviation

TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
WGA	Wheat germ agglutinin
HRP	Horseradish peroxidase
DAB	3,3'-Diaminobenzidine
PBS	Phosphate-buffered Saline
DPX	Di-N-Butyle Phthalate in Xylene
TEMED	Tetramethylethylenediamine
IAA	Iodoacetamide
DTT	Dithiothreitol
BSA	Bovine serum albumin
GO	Gene Ontology
IPG	Immobilised pH gradient
NL	Non-linear
IEF	Isoelectric focusing
ACN	Acetonitrile
PVDF	Polyvinylidine fluoride
2D-GE	Two dimensional gel electrophoresis
SDS-Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Q-TOF	Quadrupole time-of-flight
LC	Liquid chromatography
MS	Mass spectrometry
HPLC	High performance liquid chromatography
Con A	Concavanalin A
HRP	Horse radish peroxidase
SNA	Sambucus Nigra
SAP	Streptavidin-conjugated alkaline phosphatase
NBT	Nitroblue tetrazolium
BCIP	5-bromo-4-chloro-3-indolyl-phosphate

CHAPTER 1

INTRODUCTION

Swiftlets are classified into four types of genera which are Aerodramus, Hydrochous, Schoutedenapus and Collocalia. These birds can be found in caves throughout South East Asia and the local inhabitants build specialised house structures to breed them. Members of the swift family are superficially quite similar with swallow (*Hirundo* spp.). Among the different types of swiftlets, *Aerodramus* is one of the genera that are the most precious for human beings as some of the species are producing edible and nutritious nest. Other genera that are producing the edible nest include the Collocalia Swiftlet. However, the bulk of edible bird nest traded worldwide only came from two heavily exploited species: the white-nest swiftlet (*Aerodramus fuciphagus*) and the black-nest swiftlet (*Aerodramus maximus*) due to the high quality of the nest compared to others (Koon and Cranbrook, 2002). *Aerodramus* swiftlets in many aspects are typical swifts, *i.e.* having narrow wings for fast flight, a wide gap and small reduced beak surrounded by bristles for catching insects during flight. These birds are also capable of navigating through dark caves using echolocation, a feature that is also utilised by bats (Koon and Cranbrook, 2002). The swiftlet's "sonar" consists of clicking sounds at the frequencies of 1,500 to 5,500 hertz, which are audible to the human ear and are emitted at the rate of about six times per second (Gausset, 2004).

Although swiftlets hunt for insects by relying mainly on vision, *Aerodramus* swiftlets are among the very few bird species (including *Collocalia* and *Steatornis caripensis*) to use echolocation for navigation (Fullard *et al*., 1993; Price *et al*., 2004; Thomassen and Povel, 2006). As reported by Griffin and Thompson (1982) the sensitivity of the echolocation is quite high that these birds can acoustically detect and avoid wires having a diameter as small as 6.3 mm stretched across their flight space.

There are more than 24 species of insectivorous, echolocating swiftlets distributed around the world, but only a few species produce nests that are deemed 'edible' (Koon, 2000). What is more impressive is that their nest are build up using their glutinous saliva (Ma and Liu, 2012). According to Yeap (2002), the earliest history of recorded edible bird's nest (EBN) trading can be traced back to the year 1589. Admiral Cheng Ho sailed to South East Asia and brought back EBN from Indonesia as a gift to the Ming Dynasty's Emperor, following which, opened up the trade of this valuable nest. Some researchers stated that the trade can be traced back 1000 years ago during the Tang Dynasty (AD 618-907). EBN was a significant item in the cuisine and apothecary of the Emperors of China during the 16th century (Lau and Melville, 1994). EBN has been known to be one of the most precious food items by the Chinese for thousands of years (Goh *et al*., 2001). In traditional chinese medicine (TCM), EBN is believed to offer good effect to human health (Hobbs, 2004; Kong *et a1*., 1987; Chan, 2006). According to Koon and Cranbrook (2002), EBN is usually prepared for consumption by cooking in a double boiler with sugar producing a gastronomic delicacy often known as 'bird's nest soup' which is highly esteemed as a food tonic believed to have medicinal properties. The majority of EBN traded worldwide can be traced from two heavily exploited species, *i.e.* the white-nest swiftlet (*Aerodramus fuciphagus*) and the

black-nest swiftlet (*Aerodramus maximus*), whose habitats range from the Nicobar Islands in the Indian Ocean to sea caves in the coastal regions of Thailand, Vietnam, Indonesia, Borneo and the Palawan Islands in the Philippines (Koon, 2000; Koon and Cranbrook, 2002).

Even though there is a lot of information and traditional knowledge in terms of the nutritional goodness of EBN, there is still lack of scientific evidence to support all the claims made by the traditional practitioners. There are several glyconutrients that are essential in the nest (Kathan and Weeks, 1969) and this is the main reason for the swiftlet's valuable side products. Ng *et al*. (1986) reported on the immune-enhancing response of EBN by aiding immune cell division. Kong *et al*. (1987) discovered epidermal growth factor (EGF) like activity in 3T3 fibroblast that plays a role in cellular process that supports the theory of anti-aging effects by EBN. The composition of EBN from genus Aerodramus includes lipid, ash, carbohydrate and protein (Marcone, 2005). The nutritional compounds mediate the ganglioside distribution and structure in brain as has been suggested by Chan (2006). Further investigation by Guo *et al*., (2006) provided valid evidence on EBN preventing influenza viruses. The latest discovery by Nakagawa and co-workers showed that glycoproteins isolated from *Collocalia's* EBN is rich in proteoglygan (PG) containing non-sulphated chondroitin glycosaminoglycan (GAGs) (Nakagawa *et al*., 2007).

According to Marshall and Folley (2009), re-examination of the alimentary tract from two species of EBN swiftlet confirms the hypothesis by Bernstein in 1859 that the nest-cement is produced by salivary glands (Marshall and Folley, 2009). The morphology of the salivary glands has been studied since more than twelve decades ago to understand the feeding ability and nest production of the swiftlets (Helen *et al*., 2014; Faisol, 2014). As far known till now, the swiftlets will have enlarged salivary gland during breeding season for both sexes (Johnston, 1958; Helen, 2013; Faisol, 2014). However recently, Medway (2009) predicted that the nest production was contributed more by the male swiftlets as it was found that some of the female's salivary glands were inactive during breeding season. In-depth study by Wieruszeski *et al*., (1987) shows the secretion to be mucin-rich glycoprotein that hardens on contact with air forming into a cup shape nest. The saliva was also being used to consolidate food boluses to feed the chicks (Olsen and Joseph, 2011) for the population sustainability.

This study was mainly conducted to observe the differences between the sublingual glands of EBN swiftlets species; *Aerodramus fuciphagus* (white nest) and *Aerodramus maximus* (black nest) from Borneo, Malaysia in terms of their protein and glycoprotein profiles. EBN contains all 18 types of amino acids needed by human including sialic acid carbohydrate (Marcone, 2005) that beneficial towards human health. As all of the beneficial compounds that have been reported are found in the nest, this research focuses on the investigation of the proteins from the tissue of the swiftlet salivary gland itself instead from the nest as this may garner more information on the nutritional value of EBN, the characteristic and behavior of swiftlets that may be useful to the EBN industry and the consumers as well. Saliva collection from the oral of the bird was not possible as the amount is too low to be analysed in this study. To the best of our knowledge, there has been no proteomic study performed on this gland. It is expected that this present research would be able to provide reference for farmers, consumers and future researches. This study would also contribute towards a good understanding

of the proteins compounds in order to have a sustainable bird population and EBN industry in Malaysia.

The main objective of this study was to profile the proteins in the salivary gland of EBN swiftlet from *Aerodramus fuciphagus* and *Aerodramus maximus*.
The specific objectives of this study were:

1) to compare the morphological characteristics of Malaysia's EBN swiftlets and examination of the histomorphology of the salivary glands
2) to profile and identify the difference in proteins spots from the salivary glands of EBN swiftlets by using 2D-GE and mass spectrometry analysis
3) to develop the glycoprotein profile of EBN swiftlets salivary glands using 2D-GE and lectins-based detection methods.

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