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Protein Profile of Edible Bird's Nest Origin Kalimantan AndJava Islands Indonesia

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Abstract: *Edible bird's nest (EBN) was a health food supplement produced from saliva of swiftlet especially Collocalia fuciphaga. The main composition of EBN is protein and a key factor in nutritional value and therapy. Each EBN type has a unique protein profile on SDS-PAGE electrophoresis gel. Therefore, this study aimed to analyze the protein profile of EBN from Kalimantan and Java Island would be a reference for further study on EBN biological activity. This research were used EBN from swiftlet house in Kalimantan and Java Island. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to measure the molecular weight of the protein and analyzed using PhyElp 1,4 device. Cluster analysis was used to categorize EBN samples based on similarity of protein characteristics among samples. A total of 9 samples of EBN from Kalimantan and Java Island showed different protein bands in the range of 18 to 552 kDa. The protein bands always found were 55 to 59 and 107 to 127 kDa. Edible bird's nest from Kalimantan and Java Island were divided into 3 main clusters with 70% similarity level. These clusters were formed based on geographical similarity around the swiftlet houses that correlates directly with the availability and abundance of insects as a food source of swiftlet, so that ultimately produce protein bands with different patterns.*

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I. Introduction

Edible bird's nest (EBN) is a health food supplement produced from saliva of swiftlet especially *Collocaliafuciphaga*. Swiftlet is an aerial insectivora bird that catches insects while flying and spends most of its life flying, catching and eating insects. Forests, rice fields, grasslands and plantations are the main locations of swiftlets looking for insects. These location have different vegetation and insect populations[1], so making the macro and micro nutrient composition of EBN different. A small difference in the EBN biochemical composition might contribute to the variation in its biological activity. Previous research found that micro nutrients in EBN might be affected by season and even breeding site[2]. This happens because EBNwas produced by swiftlets whose its food comes from the local environment.

The main composition of EBN is protein and carbohydrates. Protein in EBN becomes a major part and a key factor in nutritional value and therapy. Protein content in EBNfrom Painan West Sumatra Indonesia was 55.62%[3] and protein content of EBN from Malaysia was 52.8-54.3%[4], and 61.0-66.9% from Thailand EBN[5]. Protein content was over 60%, therefore it was important to know whole the proteins and deeply analyze the common proteins to uncover the secrets of EBN. Protein analysis was performed with *Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis* (SDS-PAGE) technique. This technique separates proteins based on their molecular weight in the specific bands seen in polyacrylamide gel[6].

Researchershave proven in vitro that EBN extract could inhibit influenza A infections[7,8]. Compounds that act as major inhibitors of influenza virus infection was sialic acid especially Neu5Acα2-3Gal[7] bound to the terminal portion of glycoprotein. The sialic acid was found to be bound to a protein with a molecular weight of 106 and 128 kDa and the protein with a molecular weight was found to be more than 80% of the total protein in EBN[9]. Sialic acid was bound to two proteins of different molecular weights. The difference in molecular weight occurs due to differences in the composition between proteins and carbohydrates. In proteins with a molecular weight of 106 kDa it consists of 66% protein and 19% carbohydrates while protein with 128 kDa it consists of 60% protein and 24% carbohydrate[9]. Protein found abundant in EBN from Malaysia was a protein with a molecular weight 37–52 kDa[10] and each EBN type has a unique protein profile in SDS-PAGE electrophoresis and it was a different type ofprotein[11]. Different proteins would have different biological activities. Therefore,

this study aims to analyze protein profile onEBNfrom Kalimantan and Java islands which so it could be a reference for further studies on EBN biological activity.

II. Material And Methods

Edible Bird's Nest Extract

Edible Bird's nest used in this study was obtained from swiftlet house on Kalimantan Island (6) and Java Island (3).Sample preparation was done by cleaningEBN and dried for 16 hours at 70°C,grounded and filtered with a 600 µMin pore size mesh (30 mesh). Bird nests of 3.5 g were suspended in 100 ml of aquabides for 16 h at 5 °C, heated at 60 °C for 60 minutes, filtered using filter paper and stored at -20 °C for further use[7].

Bradford Assay for Protein Content in EBN

The protein content in EBN extract was measured by the Bradford method using BioRad Protein Assay (BioRad, France). The principle of this test is the existence of a bond between Coomasie Brilliant Blue G250 (CBBG) with protein in acidic condition. The color change occurred measured its absorbance at a wavelength of 595 nm[12] using a microplate reader. Briefly, the test begins with standard curve making using Bovine Serum Albumin, each at concentrations of 0, 0.5, 0.75, 1, 1.25 and 1.50 mg/ml. Furthermore, each 10 µl of standard protein and EBN extract were dissolved in 190 µl of Bradford solution (0.01 g CBBG in 5 ml of 95% ethanol (v/v), adding 10 ml of 85% phosphoric acid (v/v)) and homogenized. As much as 80 µl of each standard protein and EBN extract were put into a flat bottom 96 well microplate (Nunc - Denmark), homogenized and incubated at room temperature for 5 min. Absorbance was read on the Multiscan EX Colorimeter Reader (Thermo Scientific, Finland) at a wavelength 595 nm. The standard curve equation obtained was used to calculate the protein content of EBN extract. If the protein concentrationon EBN extract was too low, then concentrated, in order to obtain a uniform protein concentration so as not to give a bias results in electrophoresis gel. Increased concentrationwas done using Spin-X UF Concentrator (Corning - UK) at 15.000 rpm for 5 min.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation and measurement of molecular weight of proteins in EBN samples, performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method using TGX Stain-Free FastCast Acrylamide kit 12% (BioRad-France). Briefly, a clean glass plate was attached to a gel cassette. According to the manual, acrylamide gel (resolving and stacking gel) and running buffer were prepared and put into the tub of electrophoresis device (Mini Protean, BioRad). The EBN sample of 10 µg mixed with 10 µl sample buffer and 10 µg of solution put into the well of the gel (TGX ™ pre cast gel 12%), as well as the protein marker (26616, Thermo fisher scientific). The electrophoresis device was run at 150 volts for 50 min or when the sample dye reaches the lower limit of the cassette. The electrophoresis gelwas stained with Coomassie Brilliant Blue (CBB) R250 for 30 min, and to be washed with destaining solution until the clear gel or protein bands were clearly visible.

StatisticalAnalysis

SDS-PAGE results in the photo and analyzed of protein band pattern using PhyElp 1.4 devices[13]. Determination of the molecular weight of protein was performed using the BioMed MW Converter Tool. Protein bands were transformed into binary matrix form, analyzed by Hierarchical Cluster Analysis (HCA) Ward Linkage method to group EBN samples based on similarity of protein characteristics. This analysis uses Minitab ver. 16 and the results of analysis were presented in the dendrogram.

III. Result

SDS-PAGE Analysis of EBN Sample

Characterization of protein in edible bird's nestswere done by 12% SDS-PAGE, followed by CBB R250 staining that produces a unique protein band for each sample as shown in Fig. 1. In this Figure it was seen that there was a difference in the amount of protein band and its intensity, which shows the difference in protein content on the sample. The number of protein bands formed for each EBN sample was found to be different. Samples 1 and 4 have high intensityof protein band than other samples, and intensity of protein band was directly proportional to the protein content of the sample. This is consistent with the Bradford test results, that the protein content of samples 1 and 4 was higher than the other samples, that was 0.269 and 0.634 µg/µl, while the other EBN samples have a protein content 0.059 to 0.221 µg/µl (Table 1). Analysis of protein bands using MW Converter©, shows that the molecules weight of EBN protein ranges from 18 to 552kDa. Figure 1 shows that 76.6% of the protein molecule weight in the EBN sample were above 35 kDa.Interestingly, the protein bands that werealways present in every sample and found to be abundant were 55–59 (a), and 107–127 (b) kDa.Based on the geographical origin of EBN sample, protein band from Kalimatan island has a higherintensive than the protein band from Java island and the protein band on EBN from Java island found more than the EBN

from Kalimantan island. In addition, the protein band on EBNfrom Java island appears more homogeneous and the specific protein bands found in all samples from Java island were 56 and 108 kDa.

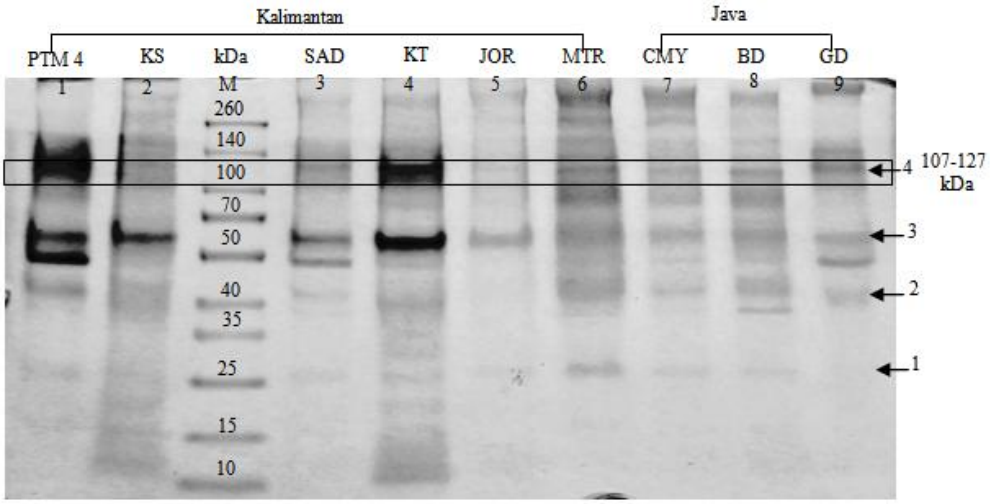

Fig 1. Protein profiles in 12% SDS-PAGE with CBB staining of 9 samples of EBN from Kalimantan and Java island. The molecular weight of the protein ranges from 18–552 kDa.

Table 1. Protein content in EBN extract from swiftlet house in Kalimatan and Java islands Indonesia

Location / Protein Content (µg/µl)								
Kalimantan Island						Java Island		
PTM 4	KS	SAD	KT	JOR	MTR	CMY	BD	GD
0.269	0.221	0.094	0.634	0.080	0.094	0.062	0.059	0.122

Cluster Analysis of EBN Sample

Cluster analysis aims to group protein profiles from SDS-PAGE based on their similarity characteristics. This analysis yields a dendrogram that wassimilarity tree of protein band pattern from EBN sample, as shown in Fig. 2. Dendrogram shows that EBN from Kalimantan and Java islands was divided into 3 main clusters with similarity level 70%. The EBN protein band from Java island grouped on cluster II that is CMY, BD and GD while EBN protein band from Kalimantan island was divided into 2 clusters namely cluster I (PTM 4, SAD and JOR) and cluster III (KS and KT). MTR protein band wasEBN from Kalimantan island, but clustered on cluster II which is EBN group from Java Island.

Cluster 1 represents EBN from the swiftlet house of SAD and JOR with similarity was higher than 70% and grouped together with PTM 4 with similarity level below 60%. The SAD swiftlet house was located in a homogeneous vegetation of palm oil and rubber plantations, while the JOR swiftlet house was located around the palm oil plantation. Cluster II represents EBN from SWY and BD swiftlet houses with similarity 100% and grouped together with MTR with similarity level close to 80% and GD with similarity close to 50%. Swiftlet house on this cluster II was generally located around the forest both primary and secondary so that clustered on the same cluster although the range of similarity value wide enough. Swiftlethouse of GD has a low similarity with other members of cluster II because of vegetation around the GD swiftlet house was a sugarcane plantation but not too far from GD swiftlet housethere were primary forest. Cluster III represents EBN from swiftlet houses KS and KT with a similarity less than 40%. Cluster III has a low similarity and in fact have vegetation of quite different. The KS swiftlet house was located in mangrove forest and coconut plantation while KT was located in mining areas and palm oil plantations.

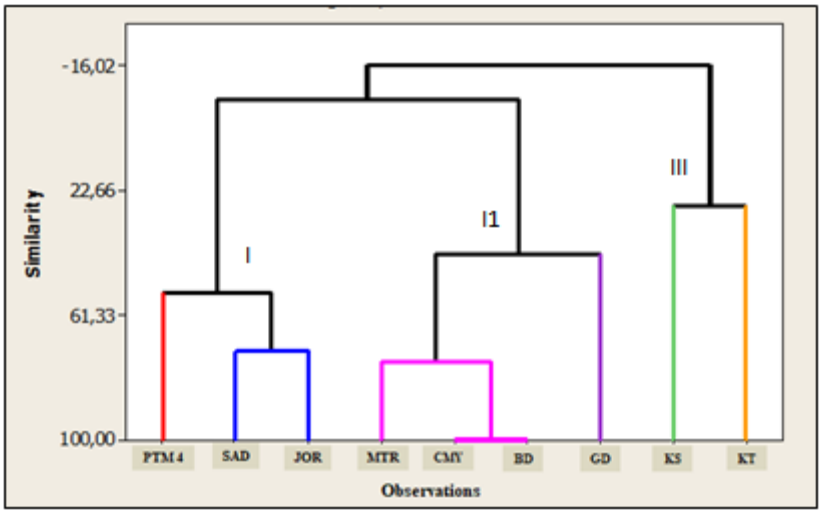

Fig 2. Dendogram hierarchy cluster analysis of word linkage method using protein profile data on SDS-PAGE from EBN samples from Kalimantan and Java island Indonesia. I, II and III show clusters of EBN that have a protein profile similarity>70%. The same line color on the dendrogram shows that the EBN were on one cluster.

IV. Discussion

Protein was a key factor in the nutritional and therapeutic value of EBN, but due to the complexity of the EBN composition, information on many proteins is unclear. Protein content in EBN reaches more than 60% dry weight. Based on the Bradfordassay, the protein content of EBN extract used in the study was less than 1%. Factor of extraction method and different of geographical location swiftlet also affect the protein content[14]. The method of protein extraction used in this research was water extraction. Water extraction method produces the highest protein content compared with other extraction methods[10]. In addition, EBN contains high amounts of water-soluble proteins and glycoproteins was seen as the main protein[15].The molecular weight range that found in this study was higher than the findings of other researchers, 18 to 552 kDa. This was probably due to extraction temperature used was not optimal to hydrolyze the protein in EBN at 70 ^{o}C for 16 hours. In addition, it might be there was no enzyme addition such as protease enzymes to hydrolyze proteins in the extraction process of EBN samples, so that the protein molecular weight found in the SDS-PAGE gels is still high. Protein extraction using high temperature by boiling EBN at 98 ± 2 °C for 8 hours then dialysis overnight using dialysis bag and then digested with pepsin A enzyme[16]. Such an extraction procedure produces protein band with molecular weight more than 200 kDa (245 kDa) for undigested samples with enzymes and 35 kDa for EBN sample digestited with pepsin enzymes.

The common protein bands in each EBN sample and the most abundant were protein bands with a molecular weight 55–59 kDa and 107–127 kDa. These protein bands were suspected proteins that dissolve in water because its protein bands have high intensity. Protein bands sufficiently broad as in samples 1 and 4 were suspected glycoproteins because previous research reports that glycoprotein was found to be quite high in EBN[15]. The protein molecular weight were found in this study slightly different withother researchers,each finding 106 and 128 kDa; 37-52 kDa and 40-70 kDa[9,10,17]. If we look at the intensity of the protein band, the EBN from Kalimantan island has a higher band intensity compared to Java island. This was an indication that the protein content of EBN from Kalimantan generally higher than EBN from Java Island. This might be caused by vegetation in Kalimantan island which better than Java island so that insects as a source of swiftletfood is also more abundant. In Fig. 1 appears that some protein bands have a smear effect. This might be due to activity of protease and oxidative enzymes and other non-protein elements[14]. Protein with lowmasswould have poor band resolution, because proteins should migrate at great distances and face higher resistance during gel migration[18].

Sialic acid were found to be bound to proteins with molecular weights 106 and 128 kDa[9],and in this study, the protein molecular weight found were not exactly the same, but still within the close value range 107–127 kDa. Researcher stated that the molecular weight that reading in the SDS-PAGE gel should not be used as a single parameter for validating a protein, because the protein migration in the SDS-PAGE gel is anomalous and the protein molecular weight was not definitive[19]. An event like this was experienced by Liu et al.[14] when identifying fragments acidic mammalian chitinase (AMCase-like). Theoretically this fragment has a molecular weight 24733.2 Da, but the real obtained in SDS-PAGE gel was 50 kDa.

The result of cluster analysis shows that the sample of EBN from Kalimantan and Java were divided into 3 clusters with similarity of 70%. Researcher states that similarities over 70% was considered high enough while under 65% was considered low for dendrograms based on protein profiles in SDS-PAGE[20]. Samples of EBN from Kalimantan island were clustered on clusters I and III, but one sample was MTR clustered on cluster II that was cluster of Java island. The EBN samples from swiftlet house of MTR clustered to Java island clusters was likely due to the similarity of vegetation, namely MTR was located in secondary forest while cluster of Kalimantan island has a homogeneous vegetation that was palm oil plantation. The EBN samples from KT swiftlet house with vegetation of palm oil plantation and mining area clustered in cluster III, not in cluster I which has the same vegetation, namely palm oil plantation and EBN from KT swiftlet house has been a bundant protein.It was suspected the sampling time of EBN at KT swiftlet house when the palm oil flowering season (anthesis). The anthesis has producesvolatile compoundsthat become one of the causal factors high abundance and diversity of insects[21].The EBN sampling from other swiftlet houses in Kalimantan were carried out when palm oil was fruitful season, so the diversity and abundance of insects was limited. The abundance and diversity of insects around the swiftlet house affect the protein content of EBN and proven on SDS-PAGE gel to produce protein band with high intensity. It was clear that the geographical environment of swiftlets greatly affects the content and diversity of proteins in EBN.

V. Conclusion

Protein content was found in EBN samples varied and there was a trend of protein content in EBN from Kalimantan island higher than Java island. The protein molecular weight found ranged from 18 to 552 kDa and as much as 76.6% of protein molecule weight in the EBN was above 35 kDa. The protein bands that were always present in each sample and found to be abundant were protein bands with molecular weight 55-59 and 107-127 kDa. Based on cluster analysis using protein profile of SDS-PAGE result, EBN from Kalimantan and Java island were divided into 3 main clusters with similarity level of 70%. This grouping based on the geographical similarity around the swiftlet house that was directly related to the diversity and abundance of insects as a food sourceof swiftlet.

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