Bioactive sialylated-mucin (SiaMuc) glycopeptide produced from enzymatic hydrolysis of edible swiftlet’s nest (ESN): degree of hydrolysis, nutritional bioavailability, and physicochemical characteristics

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ABSTRACT
The swiftlet’s nest is an edible bird’s nest (EBN) produced from dried gelatinized saliva secreted by swiftlets during the breeding season. It is widely used in industries for its high nutritional values and health benefits. However, the utilization of ESN is restricted due to its physicochemical properties, such as insolubility. The recently invented bioactive ESN hydrolyzate is reported to improve the ESN’s solubility, functional and nutritional properties to broaden its application. This study has produced bioactive ESN hydrolyzates with different hydrolysis periods to investigate the optimum conditions to achieve maximized degree of hydrolysis (DH) and to compare the physicochemical differences. The result revealed that optimum ESN hydrolysis was at 90 minutes, in which the DH and enhanced solubility (three folds) indicated a complete breakdown of glycoprotein into bioactive glycopeptide. This result is in line with the physicochemical analyses result. The hydrolysis has significantly decreased (p ≤ .05) the ESN protein content, while the levels for peptide, glycopeptide, polysaccharides, and sialic acid showed the opposite. Interestingly, the amino acid from raw ESN and hydrolyzates in all incubation periods showed no significant difference (p ≤ .05). This result suggests that the ESN biological properties remained unchanged with alcalase hydrolysis. Therefore, the findings in this study confirm the feasibility of bioactive ESN hydrolyzate as a versatile product suitable for various industries in utilizing ESN in whole instead of as extracts.

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Introduction

The edible swiftlet’s nest (ESN) or bird’s nest (EBN) is a highly valued traditional Chinese herbal material (CHM). It is a dried gelatinized salivary secretion produced by swiftlets during the breeding season. The nests are usually harvested from wild caves or dark swiftlet farm buildings with high moisture. The nests go through a cleaning process to remove feathers, dirt, dust and other inedible materials. The cleaning process usually involves soaking with water to make the ESN swell to ease the removal of foreign materials using tweezers.

There are two primary sources of consumable ESN: the white ESN are produced from Aerodramus fuciphagus, while the black ESN are from Aerodramus maximus. Traditionally, ESN is consumed by double-boiling with rock sugar and other herbal ingredients, such as ginseng, red dates, karaya gum, red seaweed, and Tremella fungus. Nowadays, many readily served ESN products in bottles and cans are available in the market. The ESN market price has been high and stable due to its limited supply and high demands worldwide. Hence, ESN is labeled as one of the most expensive animal-based protein product. This exclusiveness has even led to authenticity issues of ESN and the presence of fake swiftlet’s nests in the market.

Currently, the leading suppliers of swiftlet’s nest are Indonesia, Thailand, and Malaysia. The main market focuses on the Chinese community. The swiftlet’s nest has been widely used since the olden days, particularly in Traditional Chinese Medicine (TCM) dated as far as the Tang and Ming Dynasties of China. In the Chinese community, ESN is called “yanwo,” which means swiftlet bird’s nest. Edible swiftlet’s nest has a notable reputation and value as the “Caviar of the East” due to the belief in its medicinal and nutritional properties as a CHM in TCM. It is used in treating health disorders and consumptive diseases, such as autoimmune diseases, coughs, tuberculosis, asthma, stomach ulcer, and gastrointestinal disorders. The ESN also promotes physical and mental strength, and anti-aging to maintain youthfulness. Today, these beliefs have been proven by science for its role as a nutraceutical food. Scientific evidence supports the claims of ESN in TCM. For example, there are evidence on the ESN’s immunity enhancing properties, epidermal growth factor (EGF) that
promotes anti-aging effects\textsuperscript{[9]}, ganglioside and brain structuring by sialic acid\textsuperscript{[10]}, anti-influenza prevention properties\textsuperscript{[2]}; anti-hypertensive properties\textsuperscript{[11]}; and prebiotic ingredients that improve gut health.\textsuperscript{[12,13]}

In the modern times, ESN has been used as food supplement, nourishment, medicine, and even cosmetic component.\textsuperscript{[3,14,15]} To ensure the highly valued ESN is fully utilized, many studies have been conducted to broaden its application in a broader range of industries. However, the applications of ESN are restricted due to its physicochemical properties such as low solubility and extraction rate.\textsuperscript{[16]} For instance, the swiftlet’s nest would swell like a sponge without any further changes even after many hours or days of heating and soaking.\textsuperscript{[17]} Thus, the applications of ESN in industries such as the pharmaceutical and cosmetic industries are restricted to only utilizing the ESN extract as a functional ingredient. This deficiency limits ESN from achieving its full potential for functionality and health benefits.

In recent years, downstream processing and research on ESN development have produced a patented product known as the bioactive ESN hydrolyzate.\textsuperscript{[18,19]} This eco-friendly biotechnology has been applied on many plants and animal proteins to produce bioactive peptide (BAP) from milk, casein, whey, egg, meat, fish (e.g. atlantic cod and atlantic salmon), soy beans, sesame bran and oil by-products, peas, and even food waste like fish skin.\textsuperscript{[20-28]} There are also glycopeptide derived from commercial bovine whey protein,\textsuperscript{[29]} Ganoderma lucidum,\textsuperscript{[30]} and horseradish peroxidase.\textsuperscript{[31]} This protein enhancement as bioactive hydrolyzate allows industries to make the most of underutilized protein, protein by-products, and even specifically utilized protein with specific peptide sequences or amino acid residues.\textsuperscript{[22]} The ESN micro-particles invented by Babji in 2014 hydrolyzes the ESN SiaMuc-glycoprotein into simpler and soluble bioactive SiaMuc-glycopeptide and free peptides.\textsuperscript{[18]} In which, the enzyme converts insoluble protein into a soluble form that promotes a high rate of hydrolysis. In the second step, the soluble protein is hydrolyzed from a complex macro-protein form into simpler bioactive peptide. This technology is reported to enhance ESN in terms of nutrition, functionality, and solubility. As a result, more than 90% of ESN recovery is achieved. Also, the bioactivity of ESN such as the anti-hypertensive,\textsuperscript{[32]} anti-diabetic,\textsuperscript{[33]} and prebiotic activity\textsuperscript{[13]} are enhanced due to the application of enzymatic hydrolysis. In short, this enhancement improves the ESN application feasibility and allows industries to wholly utilize ESN in various products. As a versatile ingredient, a small quantity of ESN is sufficient to achieve maximum functionality and benefits.

Studies conducted on bioactive ESN hydrolyzate discovered evidence of improvement as an anti-hypertension,\textsuperscript{[11]} anti-oxidative,\textsuperscript{[34]} and prebiotic potential for gastrointestinal gut health,\textsuperscript{[13]} However, the effect of enzymatic hydrolysis on the physicochemical properties and nutritional bioavailability of ESN is still limited. Currently, there is only few studies regarding to hydrolysis of ESN SiaMuc-glycoprotein. That includes study of Amiza et al.\textsuperscript{[35]} on the optimization of the hydrolysis of ESN in terms of degree of hydrolysis and; study of Khushairay et al.\textsuperscript{[36]} on hydrolysis of ESN in relation to the peptide content. There is no study reported on the hydrolysis of ESN by means of degree of hydrolysis, nutritional bioavailability, and physicochemical properties. In addition, the optimization is yet to be reported, particularly on the optimized incubation period to produce ESN hydrolyzate with maximized nutritional bioavailability. Therefore, this study highlighted the production of ESN hydrolyzates with different hydrolysis periods using enzymatic biotechnology on raw ESN in order to investigate the degree of hydrolysis (DH) that determined the maximized hydrolysis period by alcalase with maximized nutritional bioavailability and functioniality. Furthermore, a comparison of nutritional bioavailability and physicochemical properties, including solubility, protein, peptide, glycoprotein/peptide, polysaccharide, reducing sugar, sialic acid, and amino acid profile of raw ESN and all hydrolyzates were also ascertained. This information may be crucial and valuable for various industries as different ESN applications require specific nutrient, criteria, and conditions.
Materials and methods

Materials

House farmed ESN samples from the swiftlet species *Aerodramus fuciphagus* were obtained from Mobile Harvester Malaysia Sdn. Bhd. in Shah Alam, Selangor, Malaysia. These samples were supplied in clean conditions as they had been subjected to a cleaning process in accordance with the company’s standard operating procedure to remove foreign materials such as feathers and other inedible impurities. The samples were crushed and ground using a homogenizer and then stored in an airtight container at room temperature for subsequent analysis. The samples were also hydrolyzed using protease from *Bacillus licheniformis* to produce ESN hydrolyzate as study objects. Samples investigated includes a double-boiled raw ESN (i.e. ER) and four different ESN hydrolyzates produced from the hydrolysis of 1, 2, 3, and 4 hours respectively (i.e. EH1, EH2, EH3, EH4). The chemicals used in this study were purchased from Sigma-Aldrich, UK.

Proximate nutritional analysis

A proximate analysis of crude protein content, fat content, ash content, moisture content and carbohydrate content was carried out in accordance with the Association of Official Analytical Chemistry methods. The proximate study was conducted before the ESN hydrolyzate production, as the hydrolysis process was protein-based content. The Kjeldahl method was used to determine the total crude protein content in ESN, using 6.25 as a conversion factor. The Soxhlet method was used to determine fat content in the sample using hexane (C6H14). The ash content was determined using “dry ashing” method at 450–550°C overnight. The ESN moisture content was determined through oven drying at 105°C for at least 16 hours until no changes of weight was detected. The carbohydrate content was obtained using the subtraction method, whereby 100% was subtracted from the total protein content, fat content, moisture content, fiber content, and ash content. The fiber content analysis was excluded as studies reported that the fiber content in ESN was insignificant with less than 0.01%. [43–45]

Sample Preparation: Double-boiling, Enzymatic Hydrolysis, and Processing of Bioactive ESN Hydrolyzate

The ESN hydrolysis was carried out based on the Babji [18] method (Patent No. WO2017034390A2). The ESN hydrolyzate production consists of the following stages: (i) Swelling process by overnight soaking, (ii) Double boiling process to facilitate the hydrolysis process, (iii) Enzymatic hydrolysis, (iv) Deactivation of hydrolysis, and (v) Freeze drying

Pre-treatment of raw ESN

The ESN double-boiling process was done according to the Garcia-Segura et al. [46] study. Raw ESN was soaked in distilled water at a ratio of 1:100 and kept overnight in a chiller at 4°C for 16 hours. Next, the soaked material was double-boiled in a water bath at 90 ± 2°C for 30 minutes. After the double-boiling process, the sample was let to cool to room temperature for enzymatic hydrolysis.

Hydrolysis of ESN

The ESN hydrolysis was carried out based on Babji’s [47] patented enzymatic process (Patent No. WO2017034390A2). The soaked ESN was placed into a pre-heated shaking water bath 15 minutes before enzyme incubation. Then, the ESN suspension was incubated with 1% alcalase enzyme at a ratio of 1:100 w/v from the total protein content. During this process, 0.1 N of NaOH was constantly added at every 15 minutes to maintain optimum pH level. The enzymatic incubation was carried out for 60,
120, 180, and 240 minutes to produce ESN hydrolyzate by four different incubation periods. After that, the hydrolysis process was deactivated with boiling water for 10 minutes. The hydrolyzate was then frozen overnight at −80°C and subsequently freeze-dried.

**Degree of hydrolysis**

The degree of hydrolysis (DH) was determined by the pH stat method from the Adler-Nissen\textsuperscript{[48]} experiment. The optimum period of ESN hydrolysis was determined when the ESN’s maximum DH was achieved. The sample’s enzymatic hydrolysis was carried out in a thermostatically controlled stirring batch reactor (Auto-titrator Horiba Scientific SZ-100). The sample’s temperature and pH were adjusted according to the enzyme requirement with 0.1 N NaOH. The hydrolysis process started when the 1% enzyme was added and the release of hydrogen in the hydrolysis process decreased the sample’s pH. The 0.1 N of NaOH was auto-titrated continuously to maintain the enzyme’s required conditions. The 0.1 N NaOH volume used during this process was recorded, and the process proceeded for 1, 2, 3, and 4 hours until a constant 0.1 N NaOH volume was reached. The DH formula was defined as the percentage ratio of the number of peptide bonds (h) broken to the total number of peptide bonds (h$_{\text{tot}}$) in the substrate.

\[
\text{DH} \, (\%) = 100 \times \frac{B}{Nb} \times \left(1/\alpha\right) \times \left(1/\text{MP}\right) \times \left(1/\text{h$_{\text{tot}}$}\right)
\]

where B is the amount of NaOH consumed (mL) during the hydrolysis; Nb is the normality of NaOH; MP is the mass (g) of the protein; α is the average degree of dissociation of the α-NH$_2$ groups released during the hydrolysis process.

\[
\alpha = 10^{(p\text{H}−pK)} / \left[1 + 10^{(p\text{H}−pK)}\right]
\]

where pK is the average dissociation value for α-amino groups liberated during hydrolysis and dependent on temperature, peptide chain length, and the nature of the terminal amino acid. The total number of peptide bonds (h$_{\text{tot}}$) in whey protein was assumed to be 8.8 meq g$^{-1}$.

**Physicochemical analysis**

*Total Solid Solubility (TSS):* The total solid solubility (TSS) test was performed by Brix refractometry method.\textsuperscript{[49]} An ESN sample solution of 2.5%, 5% and 10% (w/v) was prepared by mixing it with distilled water. A drop of the solution was placed onto the prism surface of a Brix refractometer (Mettler Toledo 0–39%) for 30 seconds. The °Brix reading was observed under bright light at room temperature and recorded as a concentration of soluble solid (%).

*Protein Content Quantification:* The Bradford method was used to measure the soluble protein content in the sample.\textsuperscript{[50]} Sample was prepared at a concentration of 1.0 mg/mL. Next, a total of 250 µL of Bradford reagent was mixed with 5.0 µL of sample and left for 2 min. The sample mixture’s absorbance was measured using an Epoch\textsuperscript{TM} Microplate Spectrophotometer (Bio Tek, US) under 595 nm wavelength. Bovine serum albumin (BSA) was used as a standard. The peptide content were measured using an o-phthaldialdehyde (OPA) spectrophotometric assay with a modified method.\textsuperscript{[11]} A fresh OPA reagent (50 mL) was prepared by mixing 25 mL of 100 mM sodium tetra-hydroborate, 2.5 mL of 20% (w/w) sodium dodecyl sulfate (SDS), 40 mg of OPA solution dissolved in 1 mL of methanol, and 100 µL of b-mercaptoethanol, and then topped up with deionized water to 50 mL. The reagent was kept in the dark to prevent degradation by light. Next, a mixture of 50 µL sample (containing 5–100 g protein) and 300 µL of OPA reagent was incubated for 2 minutes at room temperature. The sample’s absorbance was then analyzed under a spectrophotometer (Bio Tek, US) at 340 nm wavelength using L-Leucine as a standard to quantify the peptide content.
**Amino acid content**

The amino acid profile of ESN and hydrolyzate samples was analyzed using High-Performance Liquid Chromatography (HPLC) with three different hydrolysis stages prior to analysis.

**Hydrochloric acid (HCl) hydrolysis**

This analysis was performed to quantify the concentration of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine. Sample (0.2 g) was hydrolyzed with 5 mL of 6 N HCl in an enclosed tube at 110°C for 24 hours. After the sample had cooled down, 400 µL of 50 µmol α-aminobutyric acid (AABA) solution was added as an internal standard and then topped-up to 100 mL with deionized water.

**Performic acid hydrolysis**

The quantification of methionine and cysteine was done using the performic acid hydrolysis method. A sample of 0.1–0.2 g was mixed with 2 mL performic acid and stored in a chiller at 4°C for 16 hours. Next, 0.4 mL of chilled hydrogen bromide (HBr) was added, and the chilling process was continued for another 30 minutes. The sample was dried using water bath heating at 100°C, followed by HCl hydrolysis.

**Alkaline hydrolysis**

The tryptophan in the sample were quantified using alkaline hydrolysis. The sample (0.2 g) was mixed with 15 mL lithium hydroxide monohydrate (LiOH·H₂O) and heated at 120°C for 16 hours before 9 mL 6.0 N HCl was added to the sample. The sample was adjusted to pH 4.5 using 0.1 N HCl, and then topped-up with deionized water to 100 mL in a volumetric flask.

**Analysis by HPLC**

All samples were filtered using a 0.22 µm syringe filter before analysis. For the sample from the hydrochloric acid and performic acid hydrolysis, the amino acids profile was analyzed using Seo’s[51] AccQ Tag method with High-Performance Liquid Chromatography (HPLC) system (Waters 1525 Model, USA) with reverse phase of AccQ Tag Amino Acid Column (QAT052885, particle size of 4 µm, 3.9 x 150 mm). The analytes were analyzed with a fluorescence detector using 60% Acetonitrile as a mobile phase. The amount of sample injected was 10 µL at a flow rate of 1.0 mL/min with a heated column of 31–36°C isocratic elution for 12 minutes.

The tryptophan detection from the alkaline hydrolysis was conducted using a C18 Reverse phase column (Nova Pak, 5 µm, 25–30 cm) with a fluorescence detector. The system was incorporated with a mobile phase of 0.0085 M sodium acetate (pH 4.0) and methanol at a ratio of 21.7:3.3. The analysis was run at 1.0 mL/min in ambient condition with 10 µL sample injected.

**Glycan content quantification**

The phenol-sulfuric acid method was assayed to access the total polysaccharide content in sample.[52] A quantity of 1.0 mL sample (1.0 mg/mL) was mixed with 0.50 mL of 5% phenol solution (w/w), and then added with 1.5 mL concentrated sulfuric acid (H₂SO₄). The mixture was mixed well and left to set for 10 minutes at room temperature and then transferred into a 96-well microplate for analysis. The absorbance was read at 490 nm using an Epoch™ Microplate Spectrophotometer (Bio Tek, US). The standard used was 1.0 mg/mL of glucose monohydrate. The reducing sugar content was determined using the 3,5-dinitrosalicylic acid (DNS) method as described by Saqib and Whitney.[53] The DNS
reagent was prepared with 1.0 g of dinitrosalicylic acid (DNS) and 30 g of sodium potassium tartrate added into 40 mL of 2.0 N NaOH. Next, the reagent was warmed to mix well and topped-up to 100 mL with distilled water. A total volume of 1 mL sample (1.0 mg/mL) was mixed with 4 mL of DNS reagent in a test tube. The test tube sample was heated in a boiling water bath for 5 minutes, and then cooled in an ice water bath immediately. The mixture was then left to set at room temperature. The mixture’s absorbance was read using the Epoch™ Microplate Spectrophotometer (Bio Tek, US) with a wavelength of 540 nm. Glucose monohydrate was used as a standard.

**Total sialic acid content**

The resorcinol method was employed to quantify the samples’ sialic acid content. A 100 mL resorcinol reagent was prepared by mixing 0.22 g of resorcinol in 10 mL of distilled water, 80 mL of concentrated hydrochloric acid (HCl), and 0.25 mL of 0.1 M copper sulfate (CuSO₄) solution, and then topped up with distilled water. The reagent was kept in 5°C storage and became stable for analysis after 2 weeks. Sample of 500 µL was then mixed with 500 µL of resorcinol reagent in a test tube, and then covered with chilled marble for a 15-min incubation in 100°C water bath. An amount of 2.0 mL 1-butanol was then added into the solution, followed by vigorous vortex for at least 10 seconds. Next, the sample was chilled in a water bath for 10 minutes. The sample was transferred into a 96-well microplate and observed at 580 nm using Epoch™ Microplate Spectrophotometer (Bio Tek, US). Sialic acid from Sigma was used as a standard.

**Glycoprotein content**

The quantification of glycoprotein content in the sample was determined by the Periodic Acid/Schiff (PAS) method. A sample of 5 to 100 µg glycoprotein content was prepared with distilled water. Sample (2.0 mL) underwent a periodate oxidation process with 0.2 mL periodic acid solution and then incubated for 2 hours at 37°C. The periodic acid solution was prepared by mixing 10 µL of 50% (w/vol) periodic acid with 10 mL of 7% acetic acid. After the incubation, 0.2 mL of Schiff fuchsin sulfite solution was added and then incubated for 30 minutes at room temperature. The sample’s absorbance was read using the Epoch™ Microplate Spectrophotometer (Bio Tek, US) at 555 nm wavelength. Horseradish peroxidase was employed as the standard for the glycoprotein quantification.

**Antioxidative activities: DPPH, FRAP, and ABTS analysis**

**DPPH Assay:** The DPPH assay is carried out according to the study of Gan et al. DPPH reagent (0.1 mM) is prepared in methanol. Sample (100 µL) is incubated with 200 µL DPPH reagent for 30 minutes in dark, then read under a spectrophotometer (Epoch™, USA), at 517 nm. The free radical scavenging activity (%) is calculated using equation below.

\[ \text{DPPH Scavenging activity (\%)} = \frac{(A_1-A_2)}{A_1} \times 100\% \]

where \( A_1 \) is the absorbance of DPPH reagent and \( A_2 \) is the absorbance of DPPH with sample.

**Ferric reducing antioxidant power (FRAP) assay:** The FRAP assay is conducted according to the study of Al-Laith et al. FRAP reagent is prepared by mixing 300 mM sodium acetate buffer, 10 mM of 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), and 20 mM Ferum Chloride hexahydrate at a ratio of 10:1:1. Sample (250 µL) is incubated with 1.25 mL of FRAP reagent in dark for 30 minutes, then read under spectrophotometer at 595 nm. Ascorbic acid is used as standard in this assay. The FRAP value for standard and sample are calculated as ascorbic acid equivalent (AAE) per mg of sample.
ABTS Assay: The ABTS assay is performed as described in Gan et al.\textsuperscript{56} ABTS reagent is prepared by mixing 7 mM ABTS and 2.45 mM of potassium persulfate. The solution is left in dark for 12–16 hours, then diluted with methanol until an absorbance of 0.70 ± 0.02 is achieved using a spectrophotometer at 734 nm. Sample (50 µL) added with ABTS reagent (950 µL) then incubated in dark for 10 minutes before reading the absorbance. Ascorbic acid is used as standard in this assay.

Statistical analysis

All analyses were conducted in triplicates (n = 3). The one-way analysis of variance (ANOVA) was used to statistically analyze all means to determine whether there were any differences between the physicochemical properties of ESN and hydrolyzates at different hydrolysis period at 95% confidence level. The Tukey’s Multiple Range Test (p ≤ .05) was used to analyze means of treatment. The Statistical Analysis System (SAS) version 9.4 was employed as the statistical software for this study.

Result and discussion

Nutrient and composition of ESN

Table 1 shows the proximate nutritional content of a cleaned raw ESN (ER). A number of studies have found that ESN contains 80–90% of unique glycoprotein as primary component. The glycoprotein made up the 60% protein and 20–30% carbohydrate in ESN.\textsuperscript{7,43} The result in Table 1 revealed similar results. Whereby, the major nutrient of ESN is protein at 60.93%, followed by carbohydrate at 20.98%. These were also reported in previous studies.\textsuperscript{7,45,58–60} The raw ESN’s protein, carbohydrate, moisture, fat, and ash content were determined as one of the crucial procedures for the hydrolysis process. In which, the hydrolysis of ESN was performed based on the protein content in ESN.

Degree of hydrolysis and production of bioactive ESN hydrolyzate

The ESN underwent a double-boiling process to allow its structure to swell and open up to increase its bioaccessibility. The existing natural protein in food and diet is physiologically inactive within the parent protein molecule.\textsuperscript{61} Shylaja and Seshadri\textsuperscript{62} also reported a similar concept for glycoprotein, which exists in numerous forms with various functional properties in living organisms. The bioactive ESN hydrolyzate by Babji\textsuperscript{18} was developed by applying enzymatic technology to improve its micro-particulates in breaking down protein into its active form. Based on the past proximate results and Table 1, ESN has revealed a significantly low fat content.\textsuperscript{45,59} This feature was desirable for the hydrolysis process which reduces the protein-lipid complex formation affecting the production of stable protein hydrolyzate.\textsuperscript{11,63}

\[
\mu g AAE/mg = \frac{Asorbance \ of \ sample}{Asorbance \ of \ Ascorbic \ Acid} \times \ concentration \ of \ Ascorbic \ Acid \times \ Dilution \ factor
\]

Table 1. Proximate analysis of total crude protein, fat, moisture, ash, and carbohydrate in raw ESN.

| Nutritional Composition | Percentage (%) |
|-------------------------|----------------|
| Protein                 | 60.93 ± 0.60\textsuperscript{a} |
| Moisture                | 16.04 ± 1.21\textsuperscript{c} |
| Fat                     | 0.09 ± 0.01\textsuperscript{a} |
| Ash                     | 1.96 ± 0.22\textsuperscript{d} |
| Carbohydrate            | 20.98 ± 0.59\textsuperscript{b} |

\textsuperscript{a, b, c} Superscript letters show means with significant higher/lower content of nutrient composition in term of percentage (%) in ESN at a confidence level of 95% (n = 3). Note: ESN represents the edible swiftlet’s nest.
The enzymatic hydrolysis depends on the type of enzyme used that specifically cleave peptide bond in certain peptide chain and molecular weight and fractions.\textsuperscript{64,65} Example of the commercially used enzyme in the food industry is likely to be alcalse and flavourzyme which can be obtained from plant, animal and microbes. They required specific conditions such as pH, temperature, concentration, and ratio for optimum hydrolysis process.\textsuperscript{66} According to See et al.,\textsuperscript{67} alcalse is an alkaline enzyme produced from Bacillus licheniformis in brown liquid form with a slight odor. The EBN protein exists in insoluble form even undergoing physical heat treatment during the double boiling process due to complicated core structure. In the process, the EBN protein involved two procedures with first step enzyme associated to the EBN insoluble macro protein, which produces soluble peptides and amino acids, followed by the second step of which the soluble and compact core protein is being hydrolyzed again.\textsuperscript{68} The alcalse applied in EBN break its protein at a nonspecific bond. According to Adler-Nissen,\textsuperscript{48} alcalse has the highest protein recovery in protein hydrolysis compare to papain and neutrase. At the same time, the taste of the food treated with alcalse have less bitterness compares to other enzymes. This benefits in further application of the target end product.\textsuperscript{69}

Alcalse is effective in optimum condition of 60°C and pH of 8.0, while the period of hydrolysis depends on the protein characteristics and quantity. Therefore, this study produced four different bioactive ESN hydrolyzates, each with different periods of hydrolysis process at 1, 2, 3, and 4 hours. The ESN enzymatic hydrolysis released bioactive SiaMuc-glycopeptides and free peptides from its core of SiaMuc-glycoprotein and protein origin.\textsuperscript{43,70} According to Himonides et al.,\textsuperscript{71} degree of hydrolysis (DH%) of hydrolyzate is defined as the proportion of cleaved peptide bonds in a protein hydrolyzate and the key attribution for the protein hydrolyzate system. By evaluating the DH%, the hydrolytic degradation of ESN SiaMuc-glycoprotein at the different periods becomes measurable. In other words, a lower DH% meant a higher molecular weight fractions of hydrolyzates and vice versa.\textsuperscript{71} The pH-stat method used for evaluating DH% in this research detected pH changes during the hydrolysis process. The hydrolysis process broke down the SiaMuc-glycoprotein and released hydrogen ions which reduced the pH. In order to maintain the optimum pH throughout the hydrolysis process, a volume of sodium hydroxide (NaOH) was titrated for tracking trend of DH% for ESN.

Figure 1 displays an increasing ESN DH% that indicates an increasing cleaving of peptide bond and breakdown of ESN SiaMuc-glycoprotein into SiaMuc-glycopeptides during the incubation period (1–4 hours). The ESN DH% was statistically different for the first 1.5 hours (p ≤ .05). The hydrolysis rate increased vigorously for the first 1.5 hours, and then it entered a stationary phase at the next incubation period. In short, the ESN hydrolysis by alcalse had achieved its maximum rate at 1.5 hours (90 minutes).

![Figure 1](image.png)

**Figure 1.** Degree of hydrolysis (DH) for ESN hydrolyzed by alcalse for 1 hour (EH-1), 2 hours (EH-2), 3 hours (EH-3) and 4 hours (EH-4). a, b, c superscript letters shows significant higher/lower DH comparing within the period (hour) of hydrolysis for the same sample at confidence level of 95% (n = 3). Note: EH-1, EH-2, EH-3 and EH-4 represent ESN hydrolyzates with specific hydrolysis period.
A previous study by Mackie\textsuperscript{[72]} on fish protein hydrolysis reported that the DH\% rate initially decreased and eventually entered a stationary phase during incubation. Similar DH\% findings were observed in the study of ESN. Protein, being the limiting factor, affects the availability and accessibility of peptide bond for hydrolysis and eventually inhibits the production of simpler peptides and amino acids.\textsuperscript{[73]} The mechanism of the enzyme reacting toward ESN protein also influences DH\%. Based on Mohr's\textsuperscript{[68]} study on fish protein, the insoluble protein requires two steps of hydrolysis. In the first step, the enzyme converts insoluble protein into a soluble form that promotes a high rate of hydrolysis. In the second step, the soluble protein is hydrolyzed from a complex macro-protein form into simpler bioactive peptide.

The same situation is believed to have occurred for the ESN SiaMuc-glycoprotein, whereby the ESN remained in an insoluble form even after long hours of double-boiling heat treatment. Hence, the ESN's hydrolysis rate was high at the beginning and then gradually decreased. It was also observed that the ESN hydrolyzate has a lower DH\% value (~4.8–6.0\%) at the beginning of the hydrolysis process (i.e., EH-1 and EH-2) compared to the end (~10.0–12.0\%). This is related to the abundance of substrate in the initial process. The high substrate concentration reduced water availability in the hydrolysis system and diffusion process, causing the substrate to become aggregated and inhibit the hydrolysis process.\textsuperscript{[74]} In short, the ESN hydrolysis reached its optimum state at 90 minutes with a DH \% of approximately 10–11\% by enzyme alcalase in optimum conditions.

**Solubility of raw ESN and its improvement as ESN hydrolyzate**

Figure 2 illustrates the total solid solubility of raw ESN (ER) and hydrolyzates of different hydrolysis period. The investigation is performed in three (3) different concentration (i.e. 2.5\%, 5\%, and 10\% wt/vol) for accuracy. Figure 3 depicts ER's cloudy appearance after soaking and heating during the double-boiling process. The exact solution for this condition is the soluble ESN hydrolyzate. The total solid solubility (TSS) in Figure 2 shows that ER has the lowest TSS value compared to ESN hydrolyzates. The double-boiling process in ER caused the insoluble ESN SiaMuc-glycoprotein to become partially soluble with the release of bioactive SiaMuc-glycopeptide and peptide. According to Halimi et al.,\textsuperscript{[75]} the ESN solubility increases with temperature and time. Solubility depends on the polarity distribution of amino acids on the protein surface and the thermodynamic interaction of protein-water molecules. The ESN SiaMuc-glycoprotein binds easily with water when the protein unfolds from the tertiary and secondary structures with a higher degree of hydrophilic and hydrogen network. Through physical heat treatment, the ESN molecules unfold into its simpler forms and appear to be

![Figure 2. Total solid solubility (TSS) of raw ESN and hydrolyzates at concentration of 2.5\%, 5\% and 10.0\% (wt/vol).](image-url)

The TSS of each sample is investigated in three (3) different concentration, i.e. 2.5\%, 5\% and 10\%.

\textsuperscript{a, b, c} Superscript letters shows significantly higher/lower mean values comparing among samples for the same concentration at confidence level of 95\% (n = 3). Note: ESN represents edible swiftlet's nest, ER represents raw ESN and; EH-1, EH-2, EH-3 and EH-4 represent ESN hydrolyzates with specific hydrolysis period.
partially soluble in water. In other words, the ESN bioaccessibility is increased with heat. However, Hui Yan et al.\textsuperscript{[76]} claimed that ER does not completely dissolve in water even after hours or days of heating and soaking. It would remain in the form of a swelled sponge without any further changes. Current research also observed a similar occurrence in ER which had been soaked overnight and double-boiled for 30 minutes. It is therefore concluded that ESN is naturally insoluble in water.

This conclusion is also a generally recognized property of secretary mucins.\textsuperscript{[77,78]} Glycoprotein commonly exists with O-glycosidic properties.\textsuperscript{[62,79,80]} However, studies also reviewed that the glycan part of the glycoprotein provides water-holding capacity and assists in enzyme resistance. Thus, the glycoprotein is partially soluble and exists in high viscose state, for example, in saliva, egg white, and white plasma. As previously mentioned, ESN is produced from the swiftlets’ dried salivary mucins, which is partially soluble, and its viscose secretion. Naturally, ESN is also constructed mainly by glycoprotein.\textsuperscript{[81]} This feature explains the swelled ER after soaking and heating. It also describes the ESN hydrolysis’ ability to produce soluble hydrolyzate with a certain level of viscosity. Figure 2 reveals that the TSS of ESN hydrolyzates increases by three folds compared to ER. This relates to the protein structure, peptide, and amino acid within the ESN SiaMuc-glycoprotein. Studies by Brändén and Tooze,\textsuperscript{[82]} Creighton,\textsuperscript{[83]} Lesk,\textsuperscript{[84]} and Voet et al.\textsuperscript{[85]} support this finding. For example, simple protein and globular proteins are usually soluble or partially soluble and coagulable by heat. These proteins can be found in egg white, milk, blood serum, wheat leucosin, pea’s legumelin, and more. The same is applied for ESN sourced from secretary mucin that contain albuminous and globular proteins, which explains ESN’s solubility properties.\textsuperscript{[86]}

The protein solubility is also related to its peptides and amino acids. It also relies on the hydrophilic degree, the component folding,\textsuperscript{[87]} and the thermodynamic peptide–water interaction.\textsuperscript{[88]} The peptide solubility depends on its amino acids’ physical properties of peptide.\textsuperscript{[89,90]} Amino acids can be categorized as basic, acidic, polar, and non-polar. Polar amino acids are hydrophilic and able to form hydrogen bond network with water molecules. These characteristics explain the polar hydrophilic amino acids, peptides, and especially peptide chain with less than 5 units of amino acids that can dissolve in aqueous solution such as sterile water. Moreover, hydrophilic peptide with less than 25% of hydrophobic amino acids and more than 25% charged residues such as D, K, R, H, and E are usually soluble. Hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, lysine, serine, threonine, hydroxyproline, tyrosine, histidine, cysteine, and tryptophan are polar and able to bond with water molecules at the hydrogen ends.\textsuperscript{[91]} The hydrophilic amino acids are similar in quantity with the hydrophobic amino acids from the amino acid profile of ESN and hydrolyzates (Tables 2 and 3) at approximately 31–34%. This explains the solubility of ESN toward its biological properties.

Raw ESN and hydrolyzates reveal a similar amount of amino acids among the samples but a significant difference in solubility. This feature is related to the glycoprotein structure and the folding of protein, peptide or amino acids present within the ESN. The insoluble ER has a complex macro-protein structure and limited exposure of hydrophilic peptide and amino acids. For the ESN
hydrolyzates that underwent the enzyme hydrolysis process, the protein structure was unfolded and therefore exposed more hydrophilic peptides and amino acids that promoted the formation of hydrogen bond with water molecule, thus contributing to its solubility based on hydrolysis time. However, an absolute solubility will never be achieved due to the inhibition of peptide–peptide interaction and the existence of certain macro-glycoprotein that cannot be hydrolyzed.  

Contents of ESN and hydrolyzates

Figure 4 show that ESN and hydrolyzate contain glycoprotein, glycopeptide, protein, peptide, polysaccharide, reducing sugar, and sialic acid. The assays were accessed based on 1.0 mg of sample at 1.0% mixture with water. Among these contents, glycoprotein, glycopeptide, and peptide have higher proportions, making them the primary content in ESN and hydrolyzates.

Glycoprotein

The ESN glycoprotein content is quantified by the periodic acid Schiff (PAS) method, and the result is shown in Figure 4. The PAS method is widely used in quantification of macro-polysaccharides compound such as glycoprotein and glycopeptide. The PAS reagent oxidizes glyco-compound with periodic acid and stains with Schiff reagent, which creates a magenta pink liquid. In Figure 4, raw ESN (ER) has the lowest glycoprotein content (p ≤ .05). This is related to ER’s insolubility that limits glycoprotein accessibility. Studies reported that glycoprotein accessibility is influenced by the solubility, polarity, and thermodynamic interaction. For ER, the thermodynamic interaction in the overnight soaking and double-boiling process allowed the ESN structure to open up and swell in water, thus increasing the bioaccessibility of the glycoprotein content in ESN. However, a low glycoprotein content was observed due to the incomplete expression of complex macro-glycoprotein in the insoluble ER. For ESN hydrolyzates, the glycoprotein content decreased as the hydrolysis period increased. Additionally, EH-1 exhibited the highest glycoprotein content compared to EH-2, EH-3, and EH-4. This is because EH-1 underwent an incomplete hydrolysis and created a longer chain soluble
glycoprotein and glycopeptide. In contrast, EH-3 and EH-4 experienced a maximized hydrolysis state, therefore contain a higher quantity of glycopeptide instead of glycoprotein. A similar result was also obtained by Khushairay et al.\textsuperscript{[19]}

As the hydrolysis period increases, the glycopeptide content overtakes the glycoprotein content due to the complete hydrolysis process. Hur et al.\textsuperscript{[93]} claimed that the strength of glycosidic linkage in glycoprotein and glycopeptide determined the strength of the compound. The increasing hydrolysis period in EH-2, EH-3 and EH-4 that has reduced the chain length, and the structure of glycoprotein/peptide has weakened the glycosidic bonding within the glycoprotein/peptide. Thus, the glycoprotein content in ESN hydrolyzates decreased with an increasing hydrolysis period. In addition, the PAS assay was considered to be efficient for the quantification of glycoprotein as a macro-molecule of polysaccharides instead of the glycopeptide. Nevertheless, the Schiff reagent binding was less compatible to the glycopeptide due to low molecular weight (MW), simpler peptide structure, shorter peptide chain length, and further attached with large glycan molecule.\textsuperscript{[94]} Thus, the decreasing glycoprotein content in Figure 4 represents a decreasing glycopeptide content albeit an increasing glycopeptide content. Conclusively, the ESN hydrolysis is directly proportional to the glycoprotein content, reflecting the effect on glycopeptide inversely. In other words, enzymatic hydrolysis increases the nutritional bioavailability of ESN in the form of SiaMuc-glycopeptide.

\textbf{Protein, peptide, and amino acid}

\textit{Soluble protein:} Previously, proximate analysis reported that the ESN protein content was approximately 60%. However, the insoluble ER in Figure 4 reveals a relatively low soluble protein content at approximately 0.011 mg/mL. This was caused by the complex hydrophobic protein structure that had trapped the protein content within its macro-molecule. Because of this, the Bradford assay could not access the carboxyl and amino group of the soluble protein. Kruger\textsuperscript{[95]} stated that the Bradford assay does not react well with free amino acid and peptide with the molecular weight (MW) lower than 3 kDa. Previous reports claimed that ESN has a protein MW of 21.2–140.8 kDa, and glycoprotein a varied MW between 64.8 and 140.8 kDa.\textsuperscript{[96]} The ESN hydrolysis process enzymatically breaks down SiaMuc-glycoprotein and protein into SiaMuc-glycopeptides and free peptides with lower MW more than 31.4 kDa.\textsuperscript{[97]} This confirms the Bradford’s efficiency in measuring protein in ESN hydrolyzates. However, these proteins and peptides exist in the form of glycoprotein and glycopeptide. Studies reported that glycosylation interferes with the Bradford assay and consequently causes protein content underestimation.\textsuperscript{[98]}

Figure 4 also illustrates a significantly higher content of soluble protein in EH-1 and EH-2 than in other samples (p ≤ .05). Contrastingly, EH-3 and EH-4 show a statistically lower protein content than ER. This result ensures the effectiveness of the enzyme hydrolysis on the ESN SiaMuc-glycoprotein. As previously mentioned, the ESN hydrolyzates solubility increased throughout the hydrolysis period. The breakdown of ESN protein into peptides improved the protein solubility and bioaccessibility of the hydrophilic protein structure and its residues. This helped in binding the Coomassie dye in the Bradford reagent, therefore increasing the detection efficiency of the protein content in EH-1 and EH-2. Based on Figure 1, ESN achieves the maximized DH at 1.5 hours (90 minutes) and above. This is in line with the decreasing trend of the soluble protein content throughout the increasing hydrolysis period, in which EH-3 and EH-4 contain glycopeptides and peptides with lower MW. Thus, a lower concentration of soluble protein content was detected due to long hydrolysis hours.

\textit{Peptide:} The peptide content of ESN and hydrolyzate is summarized in Figure 4. Overall, the ESN peptide content was relatively higher than the soluble protein content, especially after hydrolysis. Statistically, the peptide content in ER was lower than EH-1, EH-2, EH-3, and EH-4 (p ≤ .05). However, there was no significant difference found in the peptide content of EH-1, EH-2, EH-3 and EH-4 (p ≤ .05).
Based on Perez-Vilar and Hill,\textsuperscript{[99]} mucin is constructed of polypeptide chains with glycosidic linkage. It is the swiftlet’s salivary gland’s natural properties that produce the peptide in ESN. The raw ESN is usually soaked overnight and double boiled, which then expands its structure.\textsuperscript{[11,100]} The complex and unfold hydrophilic protein in ESN breaks down into simpler peptides due to physiological factors, such as water and heat.\textsuperscript{[88]} This thermodynamic breakdown of ESN SiaMuc-glycoprotein resulted in the formation of SiaMuc-glycopeptide, free peptide, and amino acids, thus positively enhancing the OPA assay accessibility. Therefore, it is undeniable that the raw ESN has a significantly lower peptide content than the hydrolyzates. Whereas, the hydrolyzates have a significantly higher peptide content because of the enzymatic treatment that has produced simpler and soluble ESN SiaMuc-glycopeptides.

Figure 4 reveals that the ESN hydrolyzate’s peptide content increased with the hydrolysis period with a trend similar to the degree of hydrolysis (DH). A similar result is also reported by Tang et al.\textsuperscript{[101]} previously. The rise was caused by the changes in glycoprotein structure, molecular weight, solubility, and properties. The solubility increased with the hydrolysis period due to the unfolding structure and the glycopeptide and peptide exposure that enhanced the amine group accessibility in the peptide content.\textsuperscript{[100,102]} Equivalently in the ESN hydrolyzate, the ESN SiaMuc-glycopeptide hydrophilic property promoted peptide–water interaction and enhanced solubility by the formation of hydrogen bonding.

Through the enzymatic technology, the physiologically inactive SiaMuc-glycoprotein was hydrolyzed and released into the bioactive ESN SiaMuc-glycopeptide and free peptide by alcalase. Similarly to the DH trend, the peptide content entered a stationary phase from 2 hours of hydrolysis at approximately 0.90 mg/mL. This is related to the achievement of maximized DH for ESN at 1.5 hours (90 minutes). The slower hydrolysis rate of 2 hours incubation and more was arguably caused by the inhibition of high substrate concentration (i.e., peptide) and the reduction of peptide bond availability and accessibility. These phenomena have led to a less increment of peptide content in EH-3 and EH-4. The sample EH-1 reviewed a slightly lower peptide content than other hydrolyzates (EH-2, EH-3 and EH-4). This can be explained with the incomplete breakdown of macro-protein in the first hour of hydrolysis that revealed a lower solubility and bioaccessibility in the OPA assay. Conclusively, the ESN peptide content was directly proportional to the hydrolysis period that reflects on the DH, solubility, and protein structure. In other words, enzymatic hydrolysis increases the nutritional bioavailability of ESN in the form of bioactive SiaMuc-glycopeptide and free peptide.

Amino acid profile: Table 2 shows that ER and hydrolyzates consist of various amino acids, including 10 types of essential amino acids (EAA) and nine non-essential amino acids (NEAA). The results also revealed that ESN and hydrolyzates have a high concentration of aspartate, serine, valine, threonine, proline, and leucine. This confirms previous findings by Wu et al.\textsuperscript{[103]} and Chua et al.\textsuperscript{[104]} Furthermore, the World Health Organization (WHO) reported that EAA is a basic necessity for human health yet cannot be produced naturally by the human body.\textsuperscript{[105,106]} Meanwhile, NEAA indicates the opposite. The NEAA usually can be naturally synthesised by the human body; therefore, they are not mandatory in our diet.

The results in Tables 2 and 3 reveal the amino acid profile of ER and ESN hydrolyzates in terms of quantity and quality. In overall, the amino acids content of ER and ESN hydrolyzates are similar. This finding indicates that the ESN alcalase hydrolysis had no alteration and treatment effect on the ESN SiaMuc-glycopeptide amino acid sequences. This is related to the specificity of alcalase that acted on the specific peptide–peptide bond, thus reserving the amino acid profile and properties of the ESN. However, it was also observed that the quantity of certain amino acids have statistically increased with hydrolysis application. These include the essential amino acids, such as valine, tyrosine, phenylalanine, lysine, leucine, and the total amount of EAA. This is in consistent with Ng et al.\textsuperscript{[107]} who reported that the ESN enzymatic hydrolysis increased the quantity of essential amino acids. In which, the enzymatic biotechnology applied and the process that involved filtration and removal of impurities thus increased the amino acids composition and purity. As ESN and hydrolyzate is a potential processing
Table 2. Amino acid profile of ESN and hydrolyzates in percentage (%).

| Amino Acids (%) | Type of Amino Acid | ER | EH-1 | EH-2 | EH-3 | EH-4 |
|-----------------|-------------------|----|------|------|------|------|
|                 |                   |    |      |      |      |      |
| (MEAN ± STD)    |                   |    |      |      |      |      |
| **ESSENTIAL AMINO ACID (EAA)** |                   |    |      |      |      |      |
| Histidine       | his               | 2.02 ± 0.09 a 2.05 ± 0.17 a | 2.02 ± 0.25 a | 2.13 ± 0.06 a | 2.15 ± 0.15 a |
| Threonine       | thr               | 3.80 ± 0.03 a 3.82 ± 0.35 a | 4.41 ± 1.34 a | 3.94 ± 0.06 a | 3.96 ± 0.21 a |
| Tyrosine        | tyr               | 3.62 ± 0.45 b 0.52 ± 0.23 c | 3.98 ± 0.44 ab | 3.93 ± 0.12 abc | 4.20 ± 0.31 |
| Valine          | val               | 4.16 ± 0.30 a 4.19 ± 0.44 a | 4.28 ± 0.40 a | 4.28 ± 0.08 a | 4.42 ± 0.32 a |
| Methionine      | met               | 0.47 ± 0.04 a 0.46 ± 0.06 a | 0.47 ± 0.06 a | 0.48 ± 0.01 a | 0.49 ± 0.04 a |
| Lysine          | lys               | 2.09 ± 0.06 a 0.20 ± 0.18 ab | 2.04 ± 0.17 b | 2.26 ± 0.08 a | 2.22 ± 0.08 a |
| Isoleucine      | ile               | 1.83 ± 0.11 a 1.82 ± 0.20 a | 1.90 ± 0.12 a | 1.85 ± 0.03 a | 1.92 ± 0.15 a |
| Leucine         | leu               | 3.83 ± 0.31 a 3.81 ± 0.40 a | 4.01 ± 0.28 a | 3.93 ± 0.07 a | 4.05 ± 0.25 a |
| Phenylalanine   | phe               | 3.54 ± 0.25 a 3.54 ± 0.38 a | 3.57 ± 0.37 a | 3.64 ± 0.07 a | 3.76 ± 0.25 a |
| Tryptophan      | trp               | 0.70 ± 0.05 ab 0.68 ± 0.03 ab | 0.54 ± 0.16 b | 0.70 ± 0.19 ab | 0.80 ± 0.20 a |
| **NON ESSENTIAL AMINO ACIDS (N-EAA)** |                   |    |      |      |      |      |
| Hydroxyproline  | hyp               | 1.28 ± 0.25 ab 1.12 ± 0.12 b | 1.12 ± 0.10 b | 1.51 ± 0.00 a | 1.14 ± 0.11 ab |
| Aspartate       | asp               | 4.71 ± 0.18 a 4.97 ± 0.46 a | 4.75 ± 0.28 a | 5.08 ± 0.15 a | 5.10 ± 0.22 a |
| Serine          | ser               | 5.05 ± 0.02 a 4.95 ± 0.72 b | 4.86 ± 0.46 b | 5.11 ± 0.10 ab | 5.15 ± 0.25 a |
| Glutamate       | glu               | 3.58 ± 0.06 b 0.73 ± 0.25 abc | 3.53 ± 0.26 c | 3.79 ± 0.09 ab | 3.86 ± 0.09 a |
| Glycine         | gly               | 2.05 ± 0.12 a 2.07 ± 0.20 a | 2.09 ± 0.23 a | 2.15 ± 0.06 a | 2.16 ± 0.11 a |
| Arginine        | arg               | 3.19 ± 0.14 a 3.06 ± 0.34 b | 3.58 ± 0.34 a | 3.68 ± 0.08 a | 3.71 ± 0.21 a |
| Alanine         | ala               | 1.43 ± 0.10 a 1.46 ± 0.11 a | 1.46 ± 0.09 a | 1.50 ± 0.03 a | 1.52 ± 0.08 a |
| Proline         | pro               | 4.08 ± 0.29 a 4.09 ± 0.42 a | 4.21 ± 0.42 a | 4.17 ± 0.02 a | 4.38 ± 0.30 a |
| Cysteic Acid    | cya               | 1.71 ± 0.00 c 1.70 ± 0.01 c | 1.79 ± 0.04 b | 1.70 ± 0.01 c | 1.95 ± 0.01 a |
| **Total**       |                   | 53.41 ± 0.15 | 53.62 ± 0.27 | 54.06 ± 0.31 | 55.79 ± 0.07 | 56.88 ± 0.18 |
| (EAA + N-EAA)   |                   |    |      |      |      |      |

ESN represents edible swiftlet’s nest, ER represents raw ESN and; EH-1, EH-2, EH-3 and EH-4 represent ESN hydrolyzates with specific hydrolysis period. a, b, c represents the mean value with significant higher/lower content of specific amino acids.

Table 3. Categories of amino acids in raw ESN and hydrolyzates.

| Categories of amino acid | Amino acids content (%) MEAN ± STD | ER | EH-1 | EH-2 | EH-3 | EH-4 |
|--------------------------|-----------------------------------|----|------|------|------|------|
|                          |                                   |    |      |      |      |      |
| **Total essential amino acids** |                                   | 26.04 ± 1.24 b | 26.09 ± 2.21 ab | 27.19 ± 3.15 a | 27.12 ± 0.65 a | 27.95 ± 1.65 a |
| **Total non-essential amino acids** |                                   | 27.37 ± 1.00 c | 27.53 ± 1.20 ab | 26.87 ± 1.46 b | 28.67 ± 0.29 a | 28.94 ± 0.94 a |
| **Total hydrophobic amino acids** |                                   | 31.20 ± 2.04 b | 31.15 ± 2.82 b | 32.67 ± 3.95 ab | 32.24 ± 0.74 ab | 33.58 ± 2.23 a |
| **Total hydrophilic amino acids** |                                   | 32.03 ± 0.12 c | 32.17 ± 0.26 bc | 32.08 ± 0.35 ab | 33.79 ± 0.09 ab | 34.20 ± 0.17 a |

ESN represents edible swiftlet’s nest, ER represents raw ESN and; EH-1, EH-2, EH-3 and EH-4 represent ESN hydrolyzates with specific hydrolysis period. a, b, c represents the mean value with significant higher/lower content of specific amino acids.

step for “consumer product” development, where the ESN hydrolyzate is consumed as a whole material, the amino acids of ESN hydrolyzates play an important role as a matrix of its nutritional functionality.

As stated in Table 4, the EAA and NEAA of ESN is equipotential with other protein sources, such as milk, beef, egg, chicken, pork, and soybean. Edible swiftlet’s nest consists of all amino acids found in animal protein with an addition of hydroxyproline and tryptophan, which is typically absent in animal-type protein. Contrary to the protein in plants, such as soybean, ESN and hydrolyzates have high concentrations of numerous amino acids. These benefits make ESN a unique source of protein because of its complete amino acid nutrients that are beneficial to health and suitable for all consumers. Therefore, the study highlighted that ESN and hydrolyzates that existed in the form of
Table 4. Amino acid profile of ESN and hydrolyzate; and other food sources.108–110

| Amino acid          | ESN and HYD | Milk | Beef | Egg | Chicken | Pork | Soy |
|---------------------|-------------|------|------|-----|---------|------|-----|
| Essential Amino Acid (EAA) |             |      |      |     |         |      |     |
| Histidine           | 2.07        | 0.00 | 5.89 | 9.20| 7.29    | 6.74 | 0.83|
| Threonine           | 3.98        | 1.01 | 3.03 | 19.50| 3.96    | 2.76 | 0.59|
| Tyrosine            | 3.85        | 1.31 | 2.13 | 14.30| 2.40    | 2.27 | 1.51|
| Valine              | 4.26        | 1.92 | 4.33 | 15.80| 5.79    | 4.05 | 2.16|
| Methionine          | 0.47        | 0.82 | 2.03 | 9.90 | 1.97    | 2.29 | 0.45|
| Lysine              | 2.16        | 1.44 | 8.05 | 27.30| 7.83    | 8.23 | 2.03|
| Isoleucine          | 1.86        | 1.38 | 3.47 | 13.40| 3.45    | 3.35 | 1.62|
| Leucine             | 3.93        | 2.86 | 7.08 | 25.20| 6.94    | 7.26 | 2.81|
| Phenylalanine       | 3.61        | 1.52 | 3.05 | 14.40| 3.01    | 3.14 | 2.06|
| Tryptophan          | 0.68        | 0.00 | 0.00 | 0.00 | 0.00    | 0.00 | 0.00|
| Total EAA            | 26.88       | 12.26| 39.06| 149.00| 42.64   | 40.09| 14.06|
| Non-Essential Amino Acids (NEAA) |         |      |      |     |         |      |     |
| Hydroxyproline      | 1.23        | 0.00 | 0.00 | 0.00| 0.00    | 0.00 | 0.00|
| Aspartate           | 4.92        | 2.53 | 2.82 | 47.30| 2.70    | 2.86 | 5.20|
| serine              | 5.00        | 0.94 | 4.06 | 27.40| 3.81    | 4.15 | 0.23|
| Glutamate           | 3.70        | 8.28 | 7.81 | 31.80| 7.11    | 7.67 | 8.80|
| Glycine             | 2.10        | 0.51 | 20.18| 8.80 | 19.20   | 18.88| 2.04|
| Arginine            | 3.50        | 0.00 | 8.66 | 29.70| 9.27    | 8.96 | 0.22|
| Alanine             | 1.47        | 0.97 | 6.93 | 5.50 | 6.11    | 6.72 | 1.55|
| Proline             | 4.18        | 3.58 | 8.17 | 11.60| 7.43    | 8.15 | 1.77|
| Cysteic Acid        | 1.77        | 0.00 | 2.31 | 5.70 | 2.92    | 2.49 | 0.69|
| Total NEAA           | 27.87       | 16.81| 60.94| 167.80| 58.55   | 59.88| 20.50|

ESN and HYD means edible swiftlet’s nest (ESN) and hydrolyzates, respectively.

glycopeptides is comparable to other pure protein sources. In fact, ESN and hydrolyzates contains specific sequence of amino acids that is bioactive particularly in antioxidant capacity.111 Aside from the amino acids, glycann part of ESN glycopeptides as a whole are also rich in sialic acid and epidermal growth factor (EGF). These key functionality components of ESN are significant value of ESN compared to other protein sources.

Table 3 also reveals that the hydrophobic and hydrophilic amino acids in ESN and hydrolyzates are similar in concentration at approximately 31–35%, respectively. The hydrophilic amino acids in ESN and hydrolyzates include arginine, asparagine, aspartate, glutamate, lysine, serine, threonine, hydroxyproline, tyrosine, histidine, cysteine, and tryptophan with polar potential that assist in hydrogen networking in water.91 This explains ESN’s swelling capacity in water and its ability to be hydrolyzed into soluble bioactive hydrolyzate. The hydrophilic amino acids sequences and concentration influenced the degree of hydrophilic of the ESN component itself. These properties are the backbone of the ESN SiaMuc-glycoprotein structure, thermodynamic interaction, solubility, and degree of hydrolysis (DH), thus further determines the application of ESN and hydrolyzates in many industries.

The amino acid profile of ESN and hydrolyzate also revealed the hydroxyproline content, which is typically found in collagen and gelatin products. In the medical field, hydroxyproline is widely applied as a diagnostic marker in bone turnover, and in treating liver fibrosis and superficial wounds. This finding supports Chua et al.,104 Klein,112 and Hascoët et al.113 who reported that ESN contains collagen. Edible swiftlet’s nest is also well known for its antioxidant properties.111,114 Studies reported that cysteine, methionine, tryptophan, histidine, tyrosine, glycine, alanine, serine, proline, and lysine are anti-oxidative amino acids.115–117 These anti-oxidative amino acids are one of the contributors for anti-oxidation ability. The presence of anti-hypertensive and anti-diabetic amino acids is also observed in ESN. They are cysteine, arginine, tryptophan, glutamate, and leucine. These amino acids weaken insulin resistibility in glucose metabolism and eventually reduce oxidative stress, decrease vascular intracellular calcium, and increase nitric oxide production, therefore enhances the endothelial
function and reduces the peripheral vascular resistance. These would cause the lowering of the blood pressure, resulting in anti-hypertensive activity.\footnote{118} This finding is in agreement with the positive result of anti-hypertensive study in ESN.\footnote{111,114} The amino acid profile also found that ESN consists of prebiotic potential amino acids, such as alanine, aspartate, glutamate, glycine, isoleucine, leucine, proline, serine, threonine, and valine.\footnote{119–122} This result substantiates recent studies on ESN and hydrolyzate as a potential prebiotic ingredient.\footnote{12,13}

**Glycan content quantification – polysaccharide, reducing sugar, and sialic acid**

**Polysaccharide:** Carbohydrate is another primary content in ESN in a relatively abundant quantity. The carbohydrate from the ESN SiaMuc-glycoprotein, also known as glycan. In which, glycoprotein is derived from the glycosylation between galactan sugar and protein that naturally exist in the swiflet’s salivary mucin.\footnote{97} Figure 4 reveals that the ESN’s polysaccharides content increased significantly with alcalase hydrolysis ($p \leq .05$), whereby ER exhibited the lowest content of carbohydrate compared to the soluble ESN hydrolyzates. Ortiz and Wagner\footnote{123} stated that protein solubility relies on the exposure of its structure. The insoluble ER has its disturbance in exposing of its hydrophilic properties and functionality which contribute in lower quantity of polysaccharide content.

The ESN also consists of N- and O-glycosylated proteins with majority sialic acid-rich O-glycoproteins.\footnote{81} According to Cole and Smith,\footnote{124} glycoproteins are bonded covalently with sugar units known as glycan, which usually appear in the form of oligosaccharides. Just like other polysaccharide, oligosaccharide is also accessible with PHS assay.\footnote{125,126} These oligosaccharides in ESN typically includes hexoses like mannose, galactose, fucose, glucose, N-acetyhexosamine, as well as sialic acid (N-acetylneuraminic acid).\footnote{104} The polysaccharide content in ESN is the contribution of the sialic acids, such as N-acetylglucosamine, N-acetylgalactosamine, oligosaccharides, and simple sugars, such as fucose and galactose. Similar results are reported in previous studies which ESN glucan consists of 9% sialic acid (N-acetylneuraminic acid); 7.2% of N-acetylgalactosamine; 5.3% of N-acetylglucosamine; oligosaccharides, 16.9% of galactose; 0.7% of fucose; and other monosaccharides, such as mannose; glucose, rhamnose; and xylose.\footnote{127,128}

For ESN hydrolyzates, the hydrolysis results in glycopeptide as primary content instead of glycoprotein. Glycan provides protection and resistance toward proteolysis.\footnote{124} The alcalase in hydrolysis of ESN is a protease that leaves the sugar part remain untouched. However, the hydrolysis process has enhanced the exposure of galacan from the complex glycoprotein structure by strengthening the thermo-dynamic water interaction without hydrolyzing the glycan-oligosaccharide. With enhanced solubility, the polysaccharides in hydrolyzates were easily accessed with less disturbance and limitations due to its shorter peptide chain post-hydrolysis. Therefore, the ESN hydrolyzate revealed a higher polysaccharide content than the ER. Conclusively, ESN hydrolysis increases the bioavailability and bioaccessibility of polysaccharide content in ESN galacan.

Reducing Sugar: The reducing sugar content of ER and hydrolyzates in Figure 4 shows a similar quantity. This is the contribution of the oligosaccharides and monosaccharides, such as glucose, galactose, and fucose within the ESN galacan. Generally, reducing sugar comprises monosaccharides such as glucose, galactose, and fructose, and also certain disaccharides, oligosaccharides, and polysaccharides. Reducing sugar with free carbonyl group (\(C = O\)) consists of reducing functionality created by free aldehyde (-COH) or free ketone group, which then contribute in the formation of carboxyl group (-COOH).\footnote{129} The reducing sugar content of ESN is brought by the keto and aldehyde group within the galacan and protein of ESN SiaMuc-glycoprotein.

According to Shylaja and Seshadri\footnote{62} and Zhao et al.,\footnote{130} glycoproteins are compounds where glycan-sugar covalently bond to protein, and the glycan part is made of oligosaccharides.\footnote{81,131} Studies reviewed that most of the oligosaccharides, monosaccharides, glucose polymer, starch, cellulose, and copolymers (e.g chitin) possess reducing chain of aldehyde or ketone group.\footnote{132,133} The glycan contains ketone and aldehyde groups within its components, thus contributing to the reducing sugar content in ER and hydrolyzates.
Studies also proposed that the five monoses in ESN also contribute to reducing sugar content due to the presence of carbonyl carbon group.\textsuperscript{134} These mannoses are the dominant monosaccharides in N-linked glycosylation of ESN. They are the sialic acids (N-acetylenuraminic acid), N-acetylgalosamine, N-acetylgalactosamine, and some minor sugars, such as mannose and galactose. Nelson et al.\textsuperscript{134} stated that sialic acid is built by oligosaccharides which consists of an aldehyde group. The same occurs with N-acetylgalactosamine and N-acetylgalactosamine. It is believed that these components also contribute to the reducing sugar content of ESN.

The amino acids in ESN also consists of both ketone and aldehyde groups. For instance, leucine and lysine are ketogenic. Isoleucine, phenylalanine, tyrosine, and tryptophan are partially ketogenic.\textsuperscript{135,136} Tillery et al.\textsuperscript{137,138} reported that amino acids are organic compounds consisting both the amino and carboxyl groups (-COOH) in which the carboxyl group consists of both carbonyl and hydroxyl groups that represent the existence of an aldehyde group (-COH) (Phschool, 2017). These ketone and aldehyde groups attach to the amino group of amino acids, therefore interfering in the DNS assay, and consequently detected as reducing sugar. Similar phenomenon is reported by Teixeira et al.\textsuperscript{139} on the interference of amino acids in DNS assay, in which tryptophan, cysteine, histidine, tyrosine, and hydroxyproline results in overestimation of reducing sugar content by 75%, 50%, 35%, 18%, and 10%; and methionine results in lowering the reducing sugar content by 5%. Glycan also contributes to the reducing sugar content of ESN.

For ESN hydrolyzates, the reducing sugar content had no significant difference throughout the hydrolysis period. The alcalase had not affected the glycan-peptide bonding but instead cleaved on the peptide–peptide bond.\textsuperscript{140} This made the glycan-oligosaccharide of ESN SiaMuc-glycopeptide remained untouched. In addition, the unchanged amino acid profile in all ESN hydrolyzates indicates that alcalase cleave specifically without breaking down the peptide into amino acids. This decrease the bioaccessibility of aldehyde and ketone group within the amino acid which interferes the DNS assay. Hence, the natural reducing sugar in ESN remained in a similar concentration regardless of the alcalase hydrolysis period.

\textit{Sialic Acid:} Figure 4 shows that the hydrolysis had significantly increased the sialic acid content in ESN (p \leq .05). According to Halimi et al.,\textsuperscript{75} the carbohydrate in ESN is contributed by sialic acid (N-acetylenuraminic acid), N-acetylgalactosamine, N-acetylgalactosamine, galactose, and fucose. Sialic acid is the primary carbohydrate component within the ESN glycan at approximately 9%. The N-acetylenuraminic acid is an amino acid bonded with acetyl and glycolyl group.\textsuperscript{141} They function mainly in the brain’s neurological and intellectual aspects.\textsuperscript{127,142}

Sialic acids exist within the glycoproteins and gangliosides of animal tissues, fungi, yeast, and some bacteria. They are mostly found bonded to the glycan part of glycoproteins. A similar phenomenon occurs for the sialic acid in ESN SiaMuc-glycoprotein, whereby the sialyl-sugar chains together with the protein molecule.\textsuperscript{104} From the result, ER revealed a significantly lower sialic acid content than the ESN hydrolyzates (i.e. EH-1, EH-2, EH-3, and EH-4). This indicates that the soaking and heating process merely expanded the ESN SiaMuc-glycoprotein structure, yet the process was less efficient in increasing the bioavailability of sialic acid within the compound.\textsuperscript{11,100} Enzymatic hydrolysis broke down the ESN SiaMuc-glycoprotein at the peptide–peptide bond without destroying the glycan, leaving it remain untouched.\textsuperscript{114,140} The glycopeptide released from hydrolysis process has a simpler structure with increased solubility, thus increasing the sialic acid’s bioaccessibility attached within the glycan.\textsuperscript{141,143} Conclusively, the hydrolysis period was directly proportional to the sialic acid content in ESN.

\textit{Antioxidative activity: DPPH, FRAP, and ABTS scavenging activity}

Figure 5 revealed that ESN and hydrolyzates has a free radical scavenging activity (DPPH) activity of 25–30%. Whereby, a significant higher DPPH activity is observed in the ESN hydrolyzate (EH1 & EH2). While, ER has a statistically lower DPPH activity. This indicates that application enzymatic action on ESN that produced ESN in the form of bioactive glycopeptides enhanced the antioxidative
The glycoprotein, activity. a, b, c superscript with unlike letters shows significantly higher/lower in comparing among samples within the same antioxidative assay at confidence level of 95%. (n = 3). Note: The assays are carried out based on 1.0 mg/mL of ESN. ESN represents edible swiftlet’s nest, ER represents raw ESN and; EH-1, EH-2, EH-3 and EH-4 represent ESN hydrolyzates with specific hydrolysis period.

Overall, the ferric reducing antioxidant power (FRAP) value of ESN significantly increased after the application of enzymatic hydrolysis. In which, ER showed the lowest FRAP value and EH1, EH2, EH3, and EH4 have a significantly higher FRAP value. Among the ESN hydrolyzates, the findings also indicates that longer hydrolysis hour increased the FRAP value of ESN. Whereby, EH4 has the highest FRAP value; and EH1 has the lowest FRAP value. This is also reported in the previous studies where enzymatic hydrolysis released and exposed more electron-riched amino acids, thus increase the FRAP value of ESN after hydrolysis.56

The results of ABTS in Figure 5 also revealed similar findings. Whereby, the ABTS scavenging activity of ESN is significantly enhanced with enzymatic hydrolysis. In terms of period, the longer period of enzymatic hydrolysis increases the ABTS activity of ESN. Thus, EH4 with 4 hours of hydrolysis incubation period revealed the highest ABTS activity at 64.02%. Meanwhile, ER showed the lowest ABTS activity due to insolubility and low bioaccessibility of hydrophilic amino acids in ESN glyco-molecules.

**Conclusion**

The application of enzymatic technology on ESN has resulted in the production of bioactive ESN hydrolyzate. Through hydrolysis, the ESN SiaMuc-glycoprotein was converted to simpler SiaMuc-glycopeptide and free peptide. The process released bioactive glycopeptide and peptide (BAGP, BAP) from its inactive core glycoprotein. The degree of hydrolysis (DH) concluded that a 60- to 90-min hydrolysis process was required to completely breakdown the ESN SiaMuc-glycoprotein to achieve maximum DH. Further hydrolysis was unnecessary due to insignificant changes in DH, solubility, protein, peptide, and glycoprotein content. Throughout the hydrolysis process, the physicochemical properties of ESN was influenced positively, which eases industry utilization. The solubility of ESN hydrolyzate (EH-1)
was significantly improved by three folds (p ≤ .05) compared to the double-boiled raw ESN (ER). The ESN with hydrolysis period of 2 hours and more has similar solubility. The ESN physicochemical properties indicated that hydrolysis decreased glycoprotein and protein content; increased the content of bioactive glycopeptide and peptide; and allowed the bioaccessibility of glycan-polysaccharide, reducing sugar, and sialic acid. The glycopeptide and peptide content was directly proportional to the hydrolysis period until a maximized DH was achieved, while the ESN hydrolyzates’ glycan (polysaccharide, reducing and sialic acid) remained in a similar quantity regardless of the hydrolysis period. Interestingly, the ER amino acid profile and hydrolyzates proposed that the ESN hydrolysis had not influenced the quantity and quality of the amino acids in ESN. In other words, the ESN hydrolyzates provided a high concentration of beneficial amino acids with high solubility and increased nutritional bioavailability and bioaccessibility. These findings confirmed that ESN hydrolyzate improves nutritional functionality and physicochemical properties for a broader range of industrial applications. As a versatile product and material, a small quantity of ESN is required for a maximized and optimized functionality; therefore, the utilization of bioactive ESN hydrolyzate is more economical and affordable compared to ER. For a preliminary investigation of ESN hydrolyzate, further understanding of ESN hydrolyzate such as mineral content, cytotoxicity study, morphological study, and structural characteristics may be helpful in terms of industrilisation, utilization, and application.

**Highlights**

- ESN was enzymatically hydrolyzed by alcalase for four different hours.
- ESN hydrolysis produces bioactive ESN SiaMuc-glycopeptide hydrolyzate (ESNh).
- Maximum degree of hydrolysis of ESN was achieved at 60 to 90 min.
- Hydrolysis of ESN increases solubility and bioavailability of its nutrients.
- Physicochemical of ESNh showed increased in glycopeptide, peptide, and sialic acid.

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(3) Jia Lin Lee: Experimentation
(4) Seng Joe Lim: Conceptualization, Supervision
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