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Quality of edible bird's nest treated by keratinolytic enzymes-based cleaning solution

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Abstract

Edible bird's nest (EBN) is one of the luxury foods that is widely used as a healthy food due to its nutritional value and therapeutic benefits. The traditional EBN washing process causes a reduction in weight and nutrient content and an increase in pollutants due to the use of hydrogen peroxide. An exploratory observation method was used to examine the quality of EBN from *Collocalia fuciphaga* before and after washing using a washing solution based on keratinolytic enzymes. There are four stages of EBN cleaning i.e. cleaning by tap water, ethanol solution, enzyme solution at room temperature and 50°C and drying at 40°C for 42 hrs. A total of 60 EBN (unclean, n = 30) and clean, n = 30) were analyzed using the AOAC method. The mineral content of Ca, Fe, K and Mg using atomic absorption spectrophotometry (AAS) except that P was measured by spectrophotometer. A carbohydrate estimation kit was used to measure glycoprotein content and the amino acid content was determined using the HPLC method. Evaluation of the cleanliness was carried out using a scoring system by semi-trained panelists. The results obtained showed that the clean EBNs were slightly yellowish-white in color, and the cleanliness of EBN before and after cleaning increased from 2.35 to 3.84. Clean EBN protein content decreased by 7.2% while total amino acids decreased from 38.51% to 32.71%. Clean EBN contains eight essential amino acids of 17.93% with high levels of leucine, valine, arginine, and threonine (2.42-2.96%). EBN's clean ash content increased from 3.7% to 7.8%. Carbohydrate content and iron in clean EBN were 39.19±0.76% and 14.35 mg/100 g dry matter, respectively. The high levels of carbohydrates and iron appear to be good sources of glycoprotein to support health and have the potential as an alternative source of iron for people with anemia. The stepwise EBN washing method using tapwater, ethanol and a washing solution based on keratinolytic enzymes can be applied to reduce weight loss and improve the quality of EBN.

1. Introduction

Keratinase (EC3.4.21/24/99.11) is part of protease that exhibits the ability to hydrolyze keratin protein (Lange *et al*., 2016) and it primarily degrades disulfide and peptide bonds of keratinous substrates. Some natural materials that contain keratine are degraded by keratinase of serine protease or metalloprotease (Gupta and Ramnani, 2006; Sahni *et al*., 2015) produced primarily by Eukarya, Bacteria, and Archaea (Intagun and Kanoksilapatham, 2017). Extracellular keratinase produced by microorganisms has extensive application in multiple sectors, including cosmetic, biomedical, food, decontaminant agents for prion proteins, waste management, fertilizer, feed, textile industry, and cleaner or detergent additives (Šnajder *et al*., 2012; Sharma and Gupta, 2016; Li, 2019). An extracellular keratinase form of *Bacillus* sp. MTS isolated from sulfuric soil is reported to be able to degrade a whole chicken feather in 24 hours, as well as sheep wool, human hair, fish scale, and cocoon. An analysis of 16S-rRNA gene codes of *Bacillus* sp. MTS showed a 96% similarity with *Bacillus cereus* (Rahayu *et al*., 2010). The crude extracts of *Bacillus* sp. MTS contain some alkaline proteases with varied molar masses and disulfide reductase that is proven to increase the capacity of pure keratinase in hydrolyzing the keratin of chicken feathers (Rahayu *et al.*, 2012). Keratinase in the crude extract of *Bacillus* sp. MTS formulated as a cleaning agent is reported to experience increased activities by organic solvents (ethanol and glycerol) and some divalent cations, i.e.,

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Ca, Mg and Mn at some concentrations. The capacity of keratinase in the cleaning agent has increased 2-3 times compared to the control for hydrolyzing human hair and cleaning feathers stuck in the edible bird's nest or EBN (Rahayu *et al.*, 2017).

Edible bird's nest (EBN) is a substance that contains high protein and is widely used as a healthy food due to its nutritional value and therapeutic benefits. EBN soup is a renowned luxury food in Asia and is claimed to improve the immune system, respiratory function, and skin complexion (Zukefli *et al*., 2017). EBN contains mainly carbohydrates, particularly glycoprotein and amino acids (Marcone, 2005; Hamzah *et al*., 2013; Shukri *et al*., 2018), also essential trace elements, such as calcium, sodium, magnesium, zinc, manganese, and iron (Norhayati *et al.,* 2010; Hamzah *et al.,* 2013). EBN is produced from the salivary glands of swiftlets within approximately 35 days in the form of a nest that the birds can use to lay their eggs (Guo *et al*., 2006; Chua and Zukefli, 2016). The process of nest formation takes a long time and makes some materials (mostly dust, feathers, and feces) attached to and contaminates the EBN. The parameters of EBN quality in the market include the level of cleanliness and color; therefore, EBN producers are striving to produce high-quality edible nests, i.e., white and clean, thorough cleaning process.

Today, the cleaning process of EBN mostly uses hydrogen peroxide (H_2O_2) solution and bleaching that potentially contaminates the environment and harms human health. Across the globe, 50% of hydrogen peroxide is used as a bleaching agent which is expected not to accumulate in the soil and the tissues of food plants due to its unstable and reactive characteristics (Abdollahi and Hosseini, 2014). Exposure to bleaching agents may harm human health and the function of the dental pulp (Oliveira *et al*., 2012), and irritate eye membrane and lungs (Halliwell *et al*., 2000), particularly the cleaning worker in EBN factories that must focus on cleaning the contaminants attached to and trapped inside the nest. Cleaning EBN is a complicated process that takes a long time. It starts by soaking the EBN in lukewarm water for a few hours to let it soften. Then, the contaminants, such as feathers, dust, and feces are removed from the EBN manually using tweezers or hands. This intricate process has made the price of clean EBN higher than the unclean one (Shukri *et al*., 2018). However, the lengthy and multi-stage cleaning process may risk EBN losing its nutrient content and weight. In addition, a traditional cleaning method poses a higher risk to the cleaning workers because they are exposed to hydrogen peroxide for too long. Therefore, an alternative cleaning agent to hydrogen peroxide is needed. Keratinolytic protease is the more favorable cleaning agent because it is safe and eco-friendly. It is expected that the enzyme solution for cleaning EBN is safer for workers without compromising EBN nutrient content and weight. This study aimed to analyze the quality of EBN cleaned with a solution made of keratinolytic enzyme of *Bacillus* sp. MTS.

2. Materials and methods

2.1 Sample collection

Raw bird nests (*Collocalia fuchiphaga*) were obtained from the collectors harvesting bird nests from the caves along the southern coast of Central Java.

2.2 Production of cleaning solution

The enzyme was produced from *Bacillus* sp. MTS by first growing the bacteria in a minimal medium that contained 0.05% NH_4Cl, 0.05% NaCl, 0.03% K_2HPO_4, 0.01% $MgCl_2.6H_2O$, and 0.6% poultry feather meal at an optimum condition for production, pH 7.5 and 37^{o}C, agitated at 100 rpm, and incubated for two days (Rahayu *et al*., 2010; Rahayu *et al*., 2012). The cell-free extract was obtained after the production medium was centrifuged at 40^{o}C, 10.000 rpm speed, for 10 mins. Approximately 1 L of cell-free extract was incorporated with additive compounds of 2% (v/v) glycerol, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $ZnCl_2$ and 5 mM $MnCl_2$ (Rahayu *et al*., 2017). The level of keratinase activities in the cleaning solution was 0.16 U/mg. Keratinase activities in the cell-free extract were measured with 1% (w/v) feather meal substrate according to Walter (1984), and hydrolysis products were determined based on the Cysteine Standard Curve. One unit of keratinase activity is measured as the total enzymes that release one µmol Cysteine/min.

2.3 Sample preparation

Five samples of raw EBN were used in every step of the cleaning process and five samples were used as the control group (without cleaning). The cleaning process was repeated three times to obtain a total of 30 raw (unclean) and 30 clean EBNs. Before and after cleaning, the EBN was weighed and documented to track weight loss data. After the cleanliness examination, both the unclean and clean EBN previously washed and dried were composited and pulverized to powder for further analysis.

2.4 Cleaning process

EBN cleaning was conducted in five steps, i.e. (1) cleaning the contaminants attached on the surface in running water, (2) soaking and agitating at 50 rpm in 15% of ethanol solution for one min, (3) dipping EBN into enzyme solution, and incubating at room

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temperature for 10 mins, and again at 50°C for 20 mins, (4) removing feathers and feces from EBN manually using forceps, (5) rinsing EBN with clean water, then draining the water, and oven drying the EBN at 40°C for 42 hrs.

2.5 Weight loss

Weight loss (%) of EBN was calculated by unclean weight by clean weight (% dry matter) and then divided by unclean weight. The result was multiplied by 100.

2.6 Cleanliness

The cleanliness of the samples tested was performed by 15 semi-trained panelists, each with 15 samples of unclean and clean EBN offered on Petri dishes. The assessment criteria for determining the quality of EBN were color and level of cleanliness. The scoring criteria were 1 (white grey), 2 (dirty), 3 (white-grey, slightly clean) 4 (white-yellow, slightly clean) and 5 (yellowish white, clean). The data obtained were subjected to the Friedman test (Siegel, 1997).

2.7 Proximate analysis

Proximate analysis (water, protein, fat, fiber and ash content) of the EBN before and after the cleaning was measured according to guidelines published by AOAC (2019). The level of carbohydrates was obtained by subtracting the percentage of crude protein, fat, fiber, and ash from 100% of dry matter. The calculation was repeated two times, and the results were expressed as the percent of dry matter (DM) basis.

2.8 Glycoprotein content

The glycoprotein of clean and unclean EBN was measured using glycoprotein carbohydrate estimation kit from Thermo Scientific Number 23260 using the periodate oxidation method at two concentrations that could detect sialic acids and monosaccharide that have two free vicinal hydroxyl groups (Hounsell *et al*., 2002) with hexose as the standard.

2.9 Total amino acids

The total amino acids (TAA) were measured by the Beckman System Gold HPLC system (Beckman Instruments, Palo Alto, CA, US), beginning with hydrolyzing the sample in 6 mL of 6M HCl. The manufacturer recommendations (Beckman Instruments, Palo Alto, CA, USA) were used to analyzed protein hydrolysates. Ion-exchange chromatography was used to separate the amino acids which were detected after the postcolumn derivatization with ninhydrin by measuring the absorbance at 570 nm. Identification and quantification of the detected amino acids were done against external standards after adjustments through regression lines (Rahayu *et al*., 2012).

2.10 Minerals analysis

Minerals content (Ca, P, Fe, K and Mg) of unclean and clean EBN was carried out by taking 1.0 g of the oven-dried nest and digested in the concentrated hydrochloric acid as described by AOAC (2019) method. The levels of Ca, Fe, K and Mg were measured by atomic absorption spectrophotometry model GBC 940 AA, except for Phosphorus (P) which was measured spectrophotometrically at 420 nm as orthophosphate. Phosphorous reacted with a vanadate-molybdate reagent to produce stable vanadimolybdi phoshoric acid with yellow-orange color (Norhayati *et al*., 2010).

3. Results and discussion

3.1 Weight loss, glycoprotein content, color and cleanliness

EBN is a food product derived from the saliva gland of swiftlets that takes 35 days to produce by the bird in natural caves or artificial holes in buildings. The lengthy process of edible nest formation and the activity of the swiftlets may contaminate the EBN, so it must be cleaned before it is sold to consumers. However, the cleaning process potentially removes part of the nutrients and compromises the quality of EBN. From the results obtained in this study, the cleaning methods for EBN using ethanol solution, enzyme incubations, and rinsing with water caused nest weight loss by 6.89% from the average weight of 8.13±0.95 g to 7.57±1.14 g (Table 1). This result was lower than that of a conventional cleaning process, e.g., soaking in water, stirring in cooking oil, and soaking in hydrogen peroxide solution. These conventional methods are reportedly responsible for 9% weight loss of EBN (Anto, 2021). Meanwhile, Hong *et al*. (2018) develop a method for cleaning EBN that includes brushing and trimming, then compare it to the conventional method. They reported that the nutrient content of EBN increased by 30% but the weight decreased by 11.75%.

Enzyme-based solution for cleaning EBN may reduce the glycoprotein content of the EBN by 3.34% (Table 1), which is slightly higher than the 2.46% reported by Utomo *et al.* (2014) using keratinolytic solution. This discrepancy is probably due to the procedure of cleaning the nest and the origin of the nest. Meanwhile, different nutrient contents in EBN may be attributed to different types of nests, breeds, climates and feed of the swiftlets (Norhayati *et al*., 2010). The EBN cleaning process by Utomo *et al*. (2014) includes relatively two short steps i.e. soaking in 40°C water for one minute washing the EBN with enzyme solution, and

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Table 1. Weight loss, glycoprotein content, color and cleanliness EBN unclean and clean (% dry matter).

Variables	Unclean	Clean	Alteration (%)
Weight (g)	8.13±0.95	7.57±1.14	(-) 6.89
Glycoprotein (%)	10.78±0.20	10.42±0.37	(-) 3.34
Cleanliness	2.35±0.15	3.84±0.32	(+) 169

(-): decrease, (+): increase.

then removing the feather and dirt. In this study, the EBN was cleaned by several solvents in a row i.e. tap water, 15% ethanol, enzymes and running water. This process was able to remove various impurities on EBN and it yielded clean EBN with high glycoprotein content.

Cleaning solution made of keratinolytic enzymes may not significantly decrease the glycoprotein content and the protein content of EBN after cleaning remains high i.e. 52.1% (Table 1). EBN protein levels reported by other researchers were around 50-65% (Marcone, 2005; Hamzah *et al.*, 2013; Zainab *et al*., 2013; Teh and Ma, 2018). In addition, heavily glycosylated chains of peptide polymer in EBN are very difficult to hydrolyze, thus, limiting the enzyme space to interact with the substrate (Gallant *et al*., 2018), and diminishing the potential decrease of glycoprotein. The decreasing glycoprotein is due to the lack of dirt stuck in the nest.

The color of raw EBN is dull white and dirty due to the large amount of dust, feces and bird feathers attached to the nest (Figure 1a) and EBN appears shiny white after cleaning by ethanol solution, (Figure 1b). In the next step, EBN was cleaned using the enzyme-based solution to produce clean and white EBN since the feathers trapped in the EBN have been successfully removed (Figure 1c). Clean and natural white EBN was obtained after drying at 40℃ for 42 hrs (Figure 1d). Panelist evaluation on the cleanliness of EBN before and after cleaning ranged between 2.35 and 3.84, with the criteria between white-grey and slightly clean to yellowish white color and clean from dirt. It showed that the cleaning process has improved the level of panelist preference for nest cleanliness by up to 169% (Table 1). Analysis of the Friedman test showed that cleaning EBN had significantly affected ($P \leq 0.05$) the cleanliness. This result is relatively similar to that of Utomo *et al*. (2014) who reported a preference rate of cleanliness of 2.1 (unclean) and 3.84 (clean). The second step of cleanliness is soaking and EBN agitation in an ethanol solution, and the third step is dipping into an enzyme solution followed by incubation at 50℃.

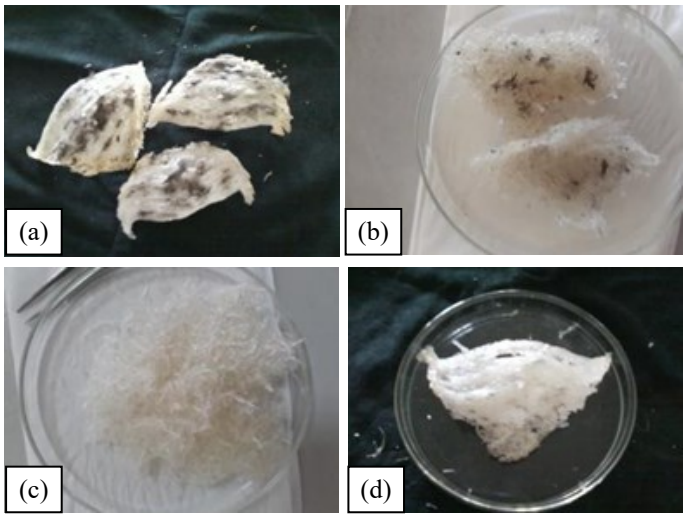

Figure 1. Edible bird's nest: (a) Unclean (Rahayu *et al*., 2017), (b) after cleaning with ethanol solution, (c) after cleaning by enzyme solution and (d) cleaned and dried.

The main goal of using ethanol solution in the cleaning process is to prepare the substrate (feather keratin stuck on EBN) for keratinase hydrolysis activity. Ethanol solution is reported to have increased keratinase activities of *Bacillus* sp. MTS from 2.2 U/mg to 4.7 U/mg (Gallant *et al*., 2018). The use of ethanol for cleaning EBN is expected to reduce the microbe population on EBN. Alcohol is a common decontamination agent to disinfect microorganisms on the surface as a preserving agents (Graziano *et al.*, 2013; Alzeer and Hadeed, 2016). The increased cleanliness of EBN has proven that ethanol and keratinolytic enzyme solution can remove dirt attached to the nest.

3.2 Nutrient composition

The proximate analysis of unclean and clean EBN using the enzyme-based solution has indicated decreasing levels of dry matter, crude protein and fat, but increasing content of crude fiber, ash and NFE (Table 2).

The contaminants attached to the EBN are mainly feces and bird feathers. A previous study reported that bird feces contained 25% protein and the feather contained 81% keratin protein (Rahayu and Bata, 2014); therefore, high protein content in unclean EBN is derived from the feces and feathers that contaminate the EBN. The cell-free extract of *Bacillus* sp. MTS contains six protease bands with molar masses of 17, 25, 32, 53, 96 and 122 kDa. While the 32 and 17 kDa are molecules with the highest keratinase activity, the 29 and 17 kDa are protein disulfide reductase (Rahayu *et al*., 2012). The stepwise cleaning process and the presence of keratinase and disulfide reductase in the cleaning agent assumedly play a vital role in hydrolyzing different contaminants attached to the EBN. The activity of keratinase hydrolysis increased by six-fold if it engaged disulfide reductase in keratin degradation of chicken feathers (Rahayu *et al*., 2012). The capacity of keratinolytic enzymes (keratinase and disulfide reductase) in the

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cleaning solution removed feces and feathers from EBN. Cleaning EBN with ethanol and enzyme solution modified the nutrient composition of unclean and clean EBN (Table 2). The present study showed decreasing levels of dry matter, protein, and fat, and increasing contents of fiber, ash and carbohydrate.

The carbohydrate content in the EBN of this study was significantly higher than that of other studies (Table 3), i.e. 35.7% (unclean) and 39.2% (clean). Carbohydrate is the second dominant component of EBN after protein, and it consists of 9.0% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose and 0.7% fructose. Sialic acid-containing sugar is the most abundant carbohydrate moiety in glycoprotein (Kathan and Weeks, 1969; Marni *et al.,* 2014). Sialic acid is essential nutrition or supplement for brain development (Wang, 2012). Chondroitin glycosaminoglycan is one of the most abundant mucinous glycoproteins, which is important for osteoporosis resistance and dermal thickness increment (Matsukawa *et al.,* 2011). EBN extract is reported to exhibit activities against the anti-avian influenza virus (Haghani *et al*., 2016) and improved immune systems (Cao *et al*., 2012). The capacity of EBN in cell proliferation has brought benefits to treatment during surgical healing, therapy for cancer treatment and against cancer recurrence (Rashed and Nazaimoon, 2010; Roh *et al.*, 2011). Hou *et al*. (2017) reported that EBN treatment for female rats for 12 weeks could improve the cognition and memory in ovariectomized. This result can be an effective option for estrogen therapy for menopause-induced aging-related memory loss. The high level of clean carbohydrates in EBN reported in this research may indicate a high level of sialic acid which is a vital substance for human health.

The presence of macro minerals like P, Mg, Ca and K in commercial EBN remains questionable because these elements cannot be detected in raw EBN (Chen *et al*., 2014). However, the present study and that of Saengkrajang *et al.* (2013) and Marcone (2005) detected the availability of those minerals in EBN. We found that the level of Fe, Mg and Ca of clean EBN has increased by 7.80%, 17.09% and 13.24%, respectively, and P increased quite significantly by 25% (Table 4). It showed that some contaminants of EBN can be removed with this cleaning method; however, contaminant removal also reduced dry matter (Table 2), thus increasing the level of Fe, Mg, Ca and P.

Table 4. Mineral content of unclean and clean EBN (mg/100 g dry matter).

EBN	Fe	P	K	Mg	Ca
Unclean	13.23	51.06	34.85	16.35	144.77
Clean	14.35	68.08	32.37	19.72	166.86
Alteration (%)	7.80	25.00	(-) 7.66	17.09	13.24

(-): decrease.

EBN is known for its rich minerals that include mainly calcium (Ca), sodium (Na), and magnesium (Mg), as well as potassium (K), phosphor (P) and iron (Fe). The levels of Mg and Fe in red EBN are reportedly higher than those in white EBN (Marcone, 2005). Compared to EBN from Thailand (Saengkrajang *et al*., 2013), the calcium and magnesium levels of clean EBN in this study are lower, i.e., 628.1-814.4 vs. 166.86 (calcium) and 142.1-148.1 vs. 19.72 (magnesium), respectively, However, the present study reported a higher level of iron in clean EBN, i.e. 14.35 vs. 0.5-1.2 mg/100 g dry matter. Different mineral contents are probably due to different cleaning treatments and the origins of EBN. The EBN from Thailand is cleaned from contaminants and feathers using forceps and scissors without any cleaning solution. High iron content is found

Table 2. Nutrient composition of clean and unclean EBN.

Edible Bird's Nest	DM (%)	% DM				
		% CP	% CF	% Fiber	% Ash	% Carbohydrate
Unclean	91.11±0.13	59.03±0.44	1.06±0.01	0.44±0.01	3.75±0.25	35.73±0.19
Clean	85.54±0.04	52.13±0.25	0.18±0.07	0.66±0.14	7.89±0.35	39.19±0.76
Alteration	(-) 6.11	(-) 11.69	(-) 83.02	(+) 50.00	(+) 110	(+) 9.68

DM: dry matter, CP: crude protein, CF: crude fat, (-): decrease, (+): increase.

Table 3. Nutrient content of clean EBN from various sources compared to this research.

Dry matter (%)	Crude protein (%)	Crude fat (%)	Carbohydrate (%)	Ash (%)	Sources
85.5	52.1	0.2	39.2	7.9	This research
79.9	53.4-53.1	0.7	-	5.7	Utomo *et al*. (2014)
88.7	62-6	0.1-1.3	25.6-27.3	2.1	Marcone (2005)
82.2-75.7	60.9-66.9	0.4-1.3	25.4-30.7	5.9-7.4	Nurul Huda *et al.* (2008)
92.9-85.1	57.9-65.8	0.01-0.09	11.0-13.0	9.5	Norrhayati *et al.* (2010); Hamzah *et al*. (2013); Zainab *et al.* (2013)
75.7-82.2	60.9-66.9	0.4-1.3	25.4-31.4	5.9-7.4	Saengkrajang *et al*. (2013)

-: no information.

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in meat products, like beef, lamb, pork, and chickens, i.e., 1.58, 1.64, 0.81, and 0.78 mg/100 g edible portions on a fresh weight basis (Petronius *et al*., 2016). High iron in clean EBN can be an alternative source of heme iron to treat anemic people with an iron deficiency which remains one of the main nutritional disorders worldwide. World Health Organization (WHO) predicted that two million people suffer from anemia throughout their life cycle, especially among pregnant women and children (Abbaspour *et al*., 2014). The level of dry matter, crude protein, fat, and ash of clean EBN in this research are close to that of EBN from Malaysian (Norhayati *et al*., 2010; Hamzah *et al*., 2013; Zainab *et al*., 2013), except the carbohydrate is higher by four times. Climate, environmental conditions, available natural feed, and the breeds of swiftlets are the potential contributing factors to different nutrient contents of EBN from a range of locations (Norhayati *et al*., 2010).

3.3 Amino acids composition

The total amino acid of EBN decreased by 15% from 38.5% (unclean) to 32.7% (clean), and the highest amino acids were aspartic acid and glutamic acid (Table 5). It is similar to Malaysian raw EBN reported by Halimi *et al.* (2014) which contained high aspartic acid (6.3%) and glutamic acid (9.6%). The present study also found relatively abundant essential amino acids, including threonine, arginine, valine, phenylalanine and leucine. The clean EBN contains eight essential amino acids (17.93%) with two least contents, i.e. methionine (0.25%) and tryptophan (undetected). Saengkrajang *et al.* (2013) performed an amino acid analysis on clean EBN from five provinces in Thailand and found that all samples did not contain tryptophan. Raw EBN from Malaysia contained the least tryptophan (0.8%) but a higher methionine (2.2%) compared to the unclean EBN. Therefore, tryptophan may be the limited amino acid in EBN.

Table 5. Amino acids composition of unclean and clean EBN (% w/w).

Amino acids	Unclean	Clean	% Decrease
Aspartic acid	4.30	3.66	14.88
Glutamic acid	3.60	2.96	17.78
Serine	3.28	2.82	14.02
Histidine	1.69	1.35	20.12
Glycine	1.81	1.47	18.78
Threonine	2.88	2.42	15.97
Arginine	3.12	2.67	14.42
Alanine	1.28	1.07	16.41
Tyrosine	2.87	2.55	11.15
Tryptophan	nd	nd	-
Methionine	0.25	0.25	0.00
Valine	3.44	2.96	13.95
Phenylalanine	3.17	2.73	13.88
Isoleucine	1.61	1.37	14.91
Leucine	3.35	2.89	13.73
Lysine	1.87	1.54	17.65
Total Amino Acids (%)	38.51	32.71	15.06

Nd: not detected.

Aspartic acid and glutamic acid are two nonessential amino acids found in EBN. These two amino acids were reported to be abundant in the lens (Ramakrishnan *et al*., 1996). According to Brosnan and Brosnan (2013), glutamic is the most abundant amino acid and plays a vital role in forming the structure, nutrition, metabolism, and signaling of protein. Glutamyl residual improves the affinity of calcium on the post-translational carboxylation and contributes significantly to hemostasis (Janke and Bulinski, 2011). While the total amino acid in clean EBN decreased by 15.06%, the branched-chain amino acids (valine, leucine, and isoleucine) decreased by 14-15% (Table 5). It indicates the effect of stepwise cleaning using water, ethanol and keratinolytic enzymes on the level of amino acids of EBN.

4. Conclusion

The stepwise cleaning of EBN using tap water, 15% ethanol solution, and keratinolytic enzymes-based solution produces clean EBN with a low weight loss, yellowish-white colour, and good level of cleanliness. The levels of dry matter, crude protein, fat, and total amino acid of clean EBN decreased but the carbohydrates increased. Higher levels of iron, P, K, Mg and Ca of clean EBN are found in clean than unclean EBN. The high iron level of clean EBN can be an alternative source of heme iron for anemic people with iron deficiency.

Conflict of interest

The authors declare no conflicts of interest.

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