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Comparative evaluation of hydrolyzed vs. un-hydrolyzed edible bird's nest across raw unclean and by-product grades

Bee-Hui Yeo[1], Teck-Kim Tang[1], Shew-Fung Wong[2], Chin-Ping Tan[3], Yaya Rukayadi[1], and Oi-Ming Lai[1,4,5]*

[1] Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia
[2] School of Medicine, International Medical University, Kuala Lumpur, Malaysia.
[3] International Joint Laboratory on Plant Oils Processing and Safety (POPS) JNU-UPM, Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Malaysia.
[4] Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Malaysia.
[5] National Institute of Fundamental Studies, Sri Lanka.
*Corresponding author: omlai@upm.edu.my

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Abstract

This study examines the impact of enzymatic hydrolysis on the nutritional properties and safety of edible bird's nest (EBN), particularly focusing on raw unclean (RUC) EBN and by-product EBN. Employing a comparative approach, the research analyzes four samples: two non-hydrolyzed (cup-shaped RUC EBN [EBNc] and by-product of EBN [EBNcp]), and two enzymatically digested (hydrolysate from EBNc [EBNch] and EBNcp [EBNcph]) using bromelain. Nutritional evaluation included measuring sialic acid content, antioxidant activity via DPPH radical scavenging, and amino acid profiling. Results indicated a significant increase in sialic acid content in the hydrolyzed samples (13.97% in EBNch and 14.36% in EBNcph) compared to non-hydrolyzed samples (12.1% in EBNc and 12.3% in EBNcp). Despite a slight reduction in antioxidant activity in hydrolyzed EBNs, they retained substantial antioxidant properties. Amino acid analysis showed the presence of leucine only in hydrolyzed samples, while isoleucine and phenylalanine were exclusive to non-hydrolyzed EBNs. Safety assessments confirmed the absence of heavy metals (arsenic, lead, mercury, cadmium, antimony) in all hydrolysates. Enzymatic hydrolysis significantly enhanced sialic acid content and specific amino acids, maintaining safety standards and proving beneficial for processing heavy feather RUC EBN and by-product EBN, thus improving product recovery and generating valuable by-products. These findings highlight the potential of enzymatic hydrolysis in enhancing the nutritional value and safety of EBN, underscoring its commercial viability.

Keywords: Edible bird’s nest, Enzymatic hydrolysis, Sialic acid, Amino acid profiling, Heavy metal contamination.

1. Introduction

Edible bird nest (EBN) is well known for its nutritional and medicinal value. Previous studies reveal the nutritional value and pharmacological activity of EBN, demonstrating the benefits of EBN. The benefits of EBN include (1) immune system enhancement and maintenance of body; (2) cell growth stimulation ; (3) anti-inflammatory effect; (4) chondro-protection against osteoarthritis and prevention against joint degeneration; (5) antioxidant capacity enhancement; (6) as an antiviral or anti-influenza agent; (7) wound healing, skin whitening, anti- aging [10]; (8) corneal wound healing promotion (eye caring) [11]; (9) advancement of stem cell proliferation; (10) multigenerational mice's learning and memory functions ; (11) neuroprotection in Alzheimer's Disease or Parkinson's Disease; (12) anti-obesity effects; (13) cardiovascular, metabolic, and diabetic illness prevention; and (14) anti-hypertensive effect [1,2].

EBN contains carbohydrates, protein, glycoprotein, moisture, fat and ash content. Compositional analysis of the purified EBN glycoprotein from previous studies showed that EBN glycoprotein contained approximately 14 % sialic acid, 63 % protein, and 21 % total saccharide [3]. Sialic acid (SA) is the signature component in EBN. Consumers always refer to the presence and percentage of sialic acid to determine the purity of EBN. SA is about 10 % in EBN [1]. A study showed that the sialic acid in EBN is only composed of N-acetylneuraminic acid (Neu5Ac). After edible bird's nest is processed by different processes (soaking, selection and high temperature sterilization), retention rate of Neu5Ac exceeds 95 %, and Neu5Ac has excellent thermal stability, and there is no significant difference ($p<0.05$) in Neu5Ac content under different conditions during the cleaning process [4]. Protein is made from twenty-plus amino acids. The nine essential amino acids (must come from food): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine [5]. Previous studies indicated that a total of 21 different amino acids (AA) were detected in EBN, but each study reported between 14 and 18 amino acids in EBN [6-14]. Lee et al. [8], Elfita et al. [14] and Babji et al. [10] showed the presence of all nine essential amino acids in the EBNs. The presence of tryptophan in EBN was only reported in these three studies. Quek et al. [6] suggested that tryptophan has not been detected because tryptophan was completely lost during acid hydrolysis for amino acid analysis. Raw unclean EBN (RUC EBN), raw clean EBN (RC EBN) and enzymatically hydrolyzed raw EBN (EBN hydrolysate) did not show significant differences ($p<0.05$) in amino acid profiles [6-14]. Heavy metal contamination in EBN has recently become a serious concern due to its relevance to food safety issues. There is a scarcity of heavy metal research, especially on EBN hydrolysate. The Malaysian Standards Department establishes the Malaysia Standard (MS) for RC EBN exported from Malaysia to China. MS 2333:2010 [Good Manufacturing Practice (GMP) For Processing Raw-Unclean and Raw-Clean EBN and MS2334:2011 EBN - Specification] are the two Malaysia Standards always use in EBN processing industry. Tolerance levels for different heavy metals related to RC EBN were obtained from MS 2334:2011. The specified limits or tolerance levels for heavy metals are: (1) Lead (Pb) $\leq$ 2 ppm; (2) Arsenic (As) $\leq$ 1 ppm; (3) Cadmium (Cd) $\leq$ 1 ppm; and (4) Mercury (Hg) $\leq$ 0.05 ppm.

Before consumption, EBN must undergo a series of treatments. Different processing processes are required for different types of EBN products. Processing technology is categorized into three categories based on technological advancements: primary processing, deep processing, and biotechnology processing, which can remove harmful substances, retain nutrients, boost flavor, and control content [2]. Primary processing (raw material: RUC EBN, product: RC EBN) is a required process for most EBN products. The process involving in the primary processing including sorting raw material, preliminary washing, softening, picking, molding, drying, sterilizing, and packaging. Picking is the most cumbersome step, and it controls the cleanliness of the EBN and is dependent on labor, thus inconsistency on product cleanliness and recovery rate from RUC EBN to RC EBN exists. Deep processing opens more markets for EBN products by expanding the variety of ready-to-eat products while reducing prices. The processing of ready-to-drink bottling EBN is the most prevalent deep processing method. The steps involved in processing ready-to-drink bottling EBN are receiving the RC EBN, remove the impurities in RC EBN, add the wet EBN in the bottle (weight of EBN is according to the manufacturer), add rock candy/ ginseng or other ingredients, cover lid of the bottle and ripen and sterilize simultaneously with autoclave/retort [2]. Other deep-processed products such as candies, jellies, beverages, effervescent tablets, nano-EBN particles, oral liquids, etc. [15-17] increase final product value through biotechnology. Biotechnology processing obtains specific nutrients in EBN primarily using extraction, enzymatic hydrolysis, separation, and other methods. Products from enzymatic hydrolysis technology (EBN hydrolysate) has broad application prospects and are widely used in EBN downstream process. Generally, due to limitations of certain physical and chemical properties (such as insolubility), the use of EBN only involves extracts rather than EBN as a whole material. Biologically active EBN hydrolysate is produced by applying enzyme technology to double-boiled EBN, which aims to decompose EBN sialylated mucin (SiaMuc) glycoprotein into simpler SiaMuc glycopeptides and free peptides [18]. In addition, this kind of biotechnology also has the characteristics of low cost (any RUC types of nests can be used), reduced processing waste, and reduced reliance on manpower. It is transformed into high-end products through biological transformation.

In recent years, there have been many enzymatic hydrolysis studies on EBN, but limited study on hydrolysate produced by-products, EBN and RUC EBN. RUC EBN is EBN harvested from swiftlet farms or cave without any cleaning process. Lower grade or heavy feathered RUC EBN used enzymatic hydrolysis to produce products without primary processing. This can save costs and may get higher product recovery. By product/ wastage EBN was insoluble residues obtained from EBN primary processing, which consists of EBN residues, dirt, feathers, and foreign matters. Enzymatic hydrolysis of by-product of EBN can potentially produce value added products. Bromelain was used in this study because it is an efficient protein-digesting enzyme and is less sensitive to pH. The low sensitivity to pH is especially important when considering applications in industry, which would reduce a step (adjustment of pH) in the process. This study aims to understand (1) the quality differences between hydrolyzed and un- hydrolyzed EBN; (2) the difference between EBN hydrolysate produced from RUC EBN and by-product EBN; and (3) safety profiles of EBN hydrolysates.

2. Materials and methods

2.1 Samples

Heavy feather cup shaped raw unclean EBN (Figure 1 - left) and by- product of EBN (Figure 1 - right) were supplied by Think Birdnest Sdn. Bhd. (Malaysia). The samples were ground into powder using mortar and pestle.

2.2 Enzymatic hydrolysis

The sample was soaked for 15 mins in water with ratio 1:20 (w/v), then water was filtered out and washed in running filtered water for 5 mins. The swelling EBN was then weighed and top up with filtered water to meet the ratio 1:20 (1g sample with 20 mL of filtered water). The sample was then double boiled at 100 °C for 30 minutes with stirring (350 rpm). Subsequently, the sample was cooled down to 50 °C before enzymatic hydrolysis. Bromelain gas dehydration unit (GDU) (2400 GDU/g) was added to the sample, according to the weight of 0.6 % of the dry weight of the EBN. Enzymatic hydrolysis was conducted for 1 hour at 50 °C with 350 rpm stirring rate. The hydrolysis was

Figure 1 Raw unclean EBN and by- product of EBN.

then filtered with a sieve to remove bigger size impurities, then sieved through smaller size WYPALL® X70 Wipers to remove tiny impurities. The hydrolysate was then double boiled for 20 min at 80 °C to denature the bromelain. The liquid hydrolysate (Figure 2) was kept at -20 °C for future work.

Figure 2 Liquid hydrolysate after enzymatic hydrolysis and filtration, free from feather and visible contamination.

2.3 Sialic acid analysis

The sialic acid analysis was conducted according to the method described by Feng et al. [19]. The 20 mg samples were dissolved in 1 mL sodium bisulfate aqueous solution (0.5 mol/L) and kept in a water bath at 80 °C for 30 minutes. Derivatization was conducted using 1mL o-phenylenediamine.2HCl as a derivative after cooling. Then, the mixture was incubated at 80 °C for another 40 minutes. After derivatization, the mixture was centrifuged, and the supernatant was collected before injecting into High Performance Liquid Chromatography

(HPLC) (Waters e2695, Milford Massachusetts, USA) for quantification at wavelength 230 nm using UV-Visible detector (Waters 2489, Milford Massachusetts, USA). Mobile phase consisting of 0.15% 1-butylamine, 0.5% phosphoric acid, acetonitrile (95:5, v/v), and 1.0% tetrahydrofuran at 1.0 mL/min flow rate was used for the chromatographic separation. The chromatographic separation was performed on a C18 column (Merck, Darmstadt, Germany).

2.4 Antioxidant activity: 2,2-diphennyl-1-picrylhydrazyl (DPPH) radical scavenging assay

A 0.036 mM DPPH reagent was prepared by dissolving 0.01407 g of DPPH powder in 1 L of methanol. EBN was soaked in distilled water for 10 minutes at a weight ratio of 1:20 (w/v). The mixture was then double boiled for an hour. After double boiling, the cooked EBN was brought to room temperature before being centrifuged for 10 minutes at 4000 rpm. The supernatant of the cooked EBN was collected. The test solution contained 14 mL of DPPH reagent and 1 mL of supernatant from the cooked EBN. The control solution used methanol instead of EBN. Both solutions were incubated for half an hour. After incubation, the test solution was filtered with a PTFE syringe filter (0.45 µm) and then measured at 517 nm using a Shimadzu UV-VIS Spectrophotometer mini 1240 (Japan). Methanol was used as the blank.

$$Free\ radical\ scavenging\ (\%) = \frac{a-b}{a} * 100$$

where a represents the DPPH control absorbance; and b represents the DPPH sample absorbance

2.5 Heavy metal analysis

Heavy metals were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Agilent, Basel, Switzerland). Samples were prepared by microwave digestion according to the standard method AOAC 999.10, 20th Ed [20]. A mixture of 1 g of EBN sample, 9 mL of concentrated nitric acid, and 2 mL of concentrated hydrochloric acid (HCl) was placed in a microwave digestion vessel. The mixture was allowed to react at room temperature for 15 minutes until the reaction subsided. A "blank" was prepared using distilled water in a digestion vessel. The digestion vessel was then covered, and microwave digestion was started. After the microwave digestion was complete, the digestion vessel was allowed to cool at room temperature for 30 minutes before opening. The container was opened, and the inside was rinsed with distilled water. The digested solution was filtered and transferred to a 25 mL volumetric flask, and the volume was brought up to the mark with distilled water. The digested solution was then analyzed by ICP-OES. The detection limit for each heavy metal was 0.01 ppm.

2.6 Amino acid profile analysis

The amino acid profiles of hydrolyzed and unhydrolyzed EBN samples were analyzed by HPLC. Samples were prepared by acid oxidation and hydrolysis and then analyzed after derivatization with phenyl isothiocyanate (PITC). The amino acid profile analysis method referred to standard method AOAC 994.12 and JAOAC, Vol 71, No 6 [21]. The sample was placed in a round-bottom flask. A mixture of 75 mg of phenol crystals, 1.5 mL of hydrogen peroxide (30%), and 13.5 mL of formic acid (88%) was then added to the sample. Samples were incubated at room temperature for 30 minutes and then in an ice box for 15 minutes. After 15 minutes, the sample was stirred at room temperature for 15 minutes. The sample was then placed in an ice box to oxidize for 16 hours. After acid oxidation, a mixture of 0.1 g of phenol crystals, 50 mL of distilled water, and 50 mL of concentrated HCl was added to the round-bottom flask. The samples were then heated at reflux for 24 hours. A 2 M sodium hydroxide solution was used to regulate the pH of the sample to 2.2. 50 mL of the standard and 50 mL of the hydrolysate were dried under nitrogen flow.

A derivatization solution was prepared by mixing 200 mL of methanol, 50 mL of distilled water, 50 mL of triethylamine, and 20 mL of PITC. Fifty microliters of the derivatization solution were added to the dried standard and hydrolysate. The samples were incubated for 20 minutes at room temperature to complete derivatization. Excess reagents were removed by drying under a stream of nitrogen. Then, 200 mL of sample diluent (0.07% w/w disodium hydrogen phosphate) was added to the dry tube. After derivatization, the mixture was injected into the HPLC and quantified using a UV-Vis detector at a wavelength of 254 nm. Chromatographic separation was achieved on a C18 column using a mobile phase containing eluent X and eluent Y at a flow rate of 1.0 mL/min. Eluent X was prepared by dissolving 19 g of sodium acetate trihydrate in 1 L of distilled water, then adding 2.5 mL of triethylamine and adjusting the pH to 6.4 with glacial acetic acid (99%). Eluent Y was prepared by mixing acetonitrile and water in a ratio of 60:40.

2.7 Statistical analysis

A *p*-value ($p < 0.05$) was calculated using Microsoft Excel (Microsoft 365, version 2395) to evaluate the statistical significance among the samples.

3. Results and discussion

3.1 Sialic acid, antioxidant activity and heavy metal in EBN samples

Figure 3 and Table 1 show the sialic acid content, antioxidant activity, and heavy metal concentration (arsenic, lead, mercury, cadmium and antimony) of four samples, two without enzymatic digestion: EBNc and EBNcp; and two enzymatically digested samples: EBNch and EBNcph. The sialic acid content was measured for all four samples: EBNc, EBNcp, EBNch, and EBNcph. EBNc, the raw unclean (RUC) EBN (EBNc) without enzymatic digestion, exhibited a sialic acid content of 12.1%. The EBN by-product sample, EBNcp, showed a sialic acid content of 12.3%. The hydrolyzed RUC sample, EBNch, demonstrated an increased sialic acid content of 13.97%. Similarly, the hydrolyzed EBN by-product sample, EBNcph, also presented an elevated sialic acid content of 14.36%. Statistical analysis revealed that EBNch and EBNcph contained significantly higher ($p < 0.05$) sialic acids than the samples without enzymatic hydrolysis (EBNc and EBNcp). Additionally, the by-product EBN samples (EBNcp and EBNcph) had higher sialic acid content compared to the raw unclean EBN samples (EBNc and EBNch), although this difference was not statistically significant ($p > 0.05$). The results indicate that enzymatic hydrolysis significantly enhances the sialic acid concentration in EBN samples. Both EBNch and EBNcph, which underwent enzymatic digestion, exhibited higher sialic acid contents (13.97% and 14.36%, respectively) compared to their non-hydrolyzed counterparts, EBNc and EBNcp (12.1% and 12.3%, respectively). This finding is consistent with the report by Ling et al. [22], which showed that EBN hydrolysate contained higher N-acetylneuraminic acid (Neu5Ac) (sialic acid) than un-hydrolyzed EBN. Additionally, the by-product EBN samples (EBNcp and EBNcph) had higher sialic acid content compared to the raw unclean EBN samples (EBNc and EBNch), though the difference was not statistically significant ($p > 0.05$). Jian-mei et al. [4] that the retention rate Neu5Ac of exceeds 95% after EBN processing.

The antioxidant activity of the four samples was evaluated using the DPPH assay. The RUC EBN sample, EBNc, exhibited an antioxidant activity of 39.39% inhibition. The by-product EBN sample, EBNcp, had an antioxidant activity of 21.84% inhibition. In contrast, the hydrolyzed sample, EBNch, showed a lower antioxidant

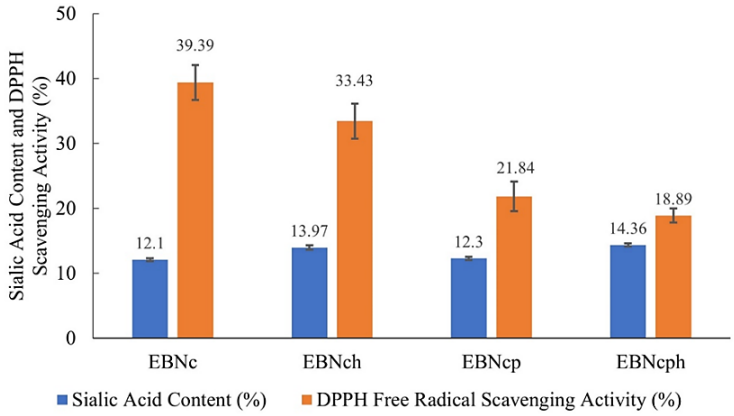

Figure 3 Sialic acid content and antioxidant activity for EBN samples.

Table 1 Heavy metal (in ppm) concentration in EBN samples.

Sample	Arsenic (As)	Lead (Pb)	Cadmium (Cd)	Mercury (Hg)	Antimony (Sb)
EBNc	N.D.	N.D.	N.D.	N.D.	N.D.
EBNcp	N.D.	N.D.	N.D.	N.D.	N.D.
EBNch	N.D.	N.D.	N.D.	N.D.	N.D.
EBNcph	N.D.	N.D.	N.D.	N.D.	N.D.

*N.D. (Not Detected) - The detection threshold is 0.01 ppm

activity of 33.43% inhibition. Similarly, the hydrolyzed by product EBN sample, EBNcph, demonstrated the lowest antioxidant activity among all samples, with 18.89% inhibition. Statistical analysis indicated that the decrease in DPPH radical scavenging activity after enzymatic hydrolysis was not significant ($p > 0.05$). The hydrolyzed samples, EBNch and EBNcph, demonstrated lower antioxidant activities (33.43% and 18.89%, respectively) compared to the non-hydrolyzed samples, EBNc and EBNcp (39.39% and 21.84%, respectively). Although the decrease in antioxidant activity after enzymatic hydrolysis was not statistically significant ($p > 0.05$), the raw unclean EBN samples exhibited significantly higher ($p < 0.05$) antioxidant activities compared to the by-product of EBN samples. These findings are consistent with previous studies, which reported a range of 8.98 – 35% for un-hydrolyzed EBN and 13.13 – 62% for hydrolyzed EBN in terms of DPPH radical scavenging activity [10, 23–25]. The values obtained in this study (18.89 - 39.39%) fall within these reported ranges.

These findings highlight the complex interplay of factors influencing the antioxidant potential of EBN and its hydrolysates. While some studies have shown that enzymatic hydrolysis can enhance the antioxidant activity of EBN [10,24], the present study observed a reduction in antioxidant activity in hydrolyzed samples (EBNch and EBNcph) compared to their non-hydrolyzed counterparts (EBNc and EBNcp). This is evident in the decrease in antioxidant activity of raw unclean EBN from 39.39% to 33.43% after hydrolysis. This unexpected decrease, although not statistically significant, might be attributed to the specific enzymes and hydrolysis conditions employed. Different enzymes cleave EBN glycoproteins at different sites, yielding a diverse range of peptides with varying bioactivities. The enzymes used in this study (bromelain) might have produced peptides with lower electron donation capabilities than the intact glycoproteins, resulting in reduced antioxidant activity. Further investigation into the peptide profiles generated by the specific hydrolysis process used is needed to elucidate this observation.

The finding that non-hydrolyzed raw unclean EBN (EBNc) displayed significantly higher antioxidant activity (39.39%) compared to non-hydrolyzed by-product EBN (EBNcp, 21.84%) is consistent with the understanding that the quality of the starting material affects the properties of the final product. RUC EBN likely contains higher levels of intact glycoproteins and other bioactive components that contribute to antioxidant activity. By-product EBN, on the other hand, might consist of degraded or lower-quality materials as it was the by- product after the primary cleaning process, leading to reduced antioxidant potential. Similarly, hydrolyzed raw unclean EBN (EBNch) maintained higher activity (33.43%) than hydrolyzed by-product EBN (EBNcph, 18.9%), further supporting the influence of the initial material quality.

Heavy metal content analysis of the samples, including lead, mercury, arsenic, cadmium, and antimony, revealed that none of these heavy metals were detected in any of the four samples studied (Table 1), which included EBN by-product and EBN hydrolysates. Previous studies have found varying levels of heavy metals in EBN samples. In Malaysia, the concentrations of lead, arsenic, and cadmium in EBN, including raw unclean EBN (RUC EBN), were always below the regulatory limits [6, 26, 27]. However, mercury levels in RUC EBNs mostly exceeded regulatory limits, although mercury in RC EBNs did not exceed these limits [6, 26, 27]. Wahyu et al. were the first to study heavy metal concentrations in EBN in Indonesia, showing that the concentrations of lead, mercury, arsenic, and cadmium for RUC EBNs and RC EBNs were always below regulatory limits [28,29]. Additionally, the study showed that washing (primary processing) the RUC EBNs can significantly reduce the heavy metals content in EBNs. It is worth noting that the heavy metal content study in Malaysia included house and cave nests, while the Indonesian study was limited to light feathered white house nests. There is limited literature on heavy metal content in EBN by-products and EBN hydrolysate. Hamzah et al. reported that lead was not present in the EBN waste (referred to here as by-products) [30]. In this study, heavy metals including arsenic, mercury, lead, cadmium, and antimony were not detected in any of the four samples, including EBN by-products and EBN hydrolysates.

Overall, the findings suggest that while enzymatic hydrolysis of EBN enhances the sialic acid content, it may reduce its antioxidant activity. The hydrolyzed samples, EBNch and EBNcph, exhibited superior sialic acid content but lower antioxidant activity compared to the non-hydrolyzed samples, EBNc and EBNcp. These results highlight the trade-offs between increasing sialic acid content and maintaining antioxidant activity in hydrolyzed EBN. Furthermore, the absence of detectable heavy metals in all samples indicates that both by-products and hydrolyzed EBN are safe for consumption in terms of heavy metal contamination. Further research is warranted to elucidate the specific mechanisms underlying these changes and to explore potential methods to retain or enhance antioxidant activity in hydrolyzed EBN for various applications in food and pharmaceutical products.

Hydrolyzed EBN has shown enhanced properties for applications in cosmetics, nutraceuticals, and functional foods due to its improved solubility, and bioavailability. For cosmetic formulations, hydrolyzed EBN peptides penetrate the skin more effectively, offering benefits such as antioxidant protection, hydration, and skin repair. In nutraceuticals and dietary supplements, the hydrolysis process increases peptide bioavailability, improving antioxidant and metabolic health properties [18, 10]. Functional food applications also benefit from hydrolyzed EBN due to its solubility and ease of incorporation into beverages and snacks [9]. In contrast, un-hydrolyzed EBN retains its intact glycoprotein structure, which is advantageous for traditional food preparations and luxury health products, especially in markets emphasizing minimal processing and cultural significance. EBN grades further

influence their applications. High-grade raw unclean EBN, with its rich glycoprotein content, and better appearance is suitable for premium supplements and foods. By-product EBN, being cost-effective, is ideal for hydrolysis to produce bioavailable hydrolysates for broader commercial use [22].

These findings highlight that hydrolyzed EBN is advantageous for modern applications, while un-hydrolyzed EBN remains valuable in traditional and premium markets. Further studies on peptide profiles from different hydrolysis methods will help optimize these applications.

3.2 Amino acid profile

Amino acid profiling of hydrolyzed and un-hydrolyzed samples was performed to determine the effect of hydrolysis on amino acid profiles and to identify the differences between the hydrolysates of different grades of EBN. Table 2 shows amino acid profile of hydrolyzed raw unclean EBN (EBNch), un-hydrolyze raw unclean EBN (EBNc), hydrolyzed by- product of EBN (EBNcph) and un-hydrolyze by- product of EBN (EBNcp).

Table 2 Amino acid profile of hydrolyzed raw unclean EBN (EBNch), un-hydrolyze raw unclean EBN (EBNc), hydrolyzed by- product of EBN (EBNcph) and un-hydrolyze by- product of EBN (EBNcp).

		Amino Acid Profile % (w/w)			
		EBNc	EBNch	EBNcp	EBNcph
Essential Amino Acids (EAAs)	Histidine	0.97	0.84	1.08	0.57
	Isoleucine	4.08	0.00	4.82	0.00
	Leucine	0.00	6.71	0.00	6.89
	Lysine	0.00	0.00	1.45	0.86
	Methionine	0.00	0.00	0.00	0.00
	Phenylalanine	2.99	0.00	3.22	0.00
	Threonine	1.87	1.68	1.95	1.72
	Tryptophan	n.q	n.q	n.q	n.q
	Valine	2.71	2.51	2.89	2.01
	Subtotal	12.62	11.74	15.41	12.06
Non- essential Amino Acids (NEAAs)	Alanine	2.18	2.24	2.34	2.01
	Arginine	4.99	4.19	4.38	2.01
	Aspartic acid	4.22	3.63	4.38	4.02
	Cysteine	n.q	n.q	n.q	n.q
	Glutamic acid	3.06	2.51	3.08	2.01
	Glycine	1.59	1.40	1.85	2.30
	Proline	1.60	1.68	1.63	1.15
	Serine	3.21	3.07	3.46	2.58
	Tyrosine	0.31	1.96	1.26	2.01
	Glutamine	n.q	n.q	n.q	n.q
	Asparagine	n.q	n.q	n.q	n.q
	Cystine	n.q	n.q	n.q	n.q
	4- hydroxyproline	n.q	n.q	n.q	n.q
	Sub Total	21.16	20.68	22.38	18.09
Total		33.78	32.41	37.79	30.15
Essential/Total		0.37	0.36	0.41	0.40

The analysis revealed that hydrolyzed EBN (EBNch and EBNcph) exhibited significant enhancements in certain amino acids compared to un-hydrolyzed forms. Notably, Leucine was only present in hydrolyzed forms, with EBNch and EBNcph containing 6.71% and 6.89% respectively, while being absent in un-hydrolyzed EBNc and EBNcp. This indicates that hydrolysis may release or preserve this essential amino acid. Although some

essential amino acids (EAAs) such as Isoleucine and Phenylalanine were absent in hydrolyzed forms, the overall profile remained rich, with EBNch and EBNcph containing total EAA concentrations of 11.74% and 12.06%, respectively, compared to 12.62% in EBNc and 15.41% in EBNcp.

Hydrolysis has several advantages in enhancing the amino acid profile of EBN. The process breaks down protein structures, making amino acids more accessible and bioavailable. This is particularly beneficial for essential amino acids like Leucine, which plays a critical role in muscle protein synthesis and recovery. Enhanced levels of Leucine in hydrolyzed EBN products can contribute to better muscle health and maintenance, which is advantageous for individuals with higher protein needs, such as athletes and the elderly. Furthermore, the presence of bioactive peptides in hydrolyzed proteins can offer additional health benefits, including antioxidant, antimicrobial, and anti-inflammatory properties. The improved digestibility of hydrolyzed proteins also means that nutrients are absorbed more efficiently, enhancing overall nutritional status and promoting gut health.

The total amino acid content in hydrolyzed by-products (EBNcph) was 30.15%, comparable to 32.41% in hydrolyzed raw unclean EBN (EBNch). The essential/total amino acids ratio was also similar between EBNch (0.36) and EBNcph (0.40), underscoring the consistent nutritional value of hydrolyzed products regardless of their origin. The percentage of amino acids after enzymatic hydrolysis decreased but not significantly ($p > 0.05$). Previous literature reported that the ratio of essential amino acids to total amino acids was in the range of 0.342-0.565 [7-14]. The rates for this study (0.36-0.41) are also within this range, demonstrating the effectiveness of hydrolysis. These findings suggest that hydrolyzed EBN products, whether derived from raw unclean EBN or by-products, offer enhanced amino acid profiles, validating the advantages of hydrolysis in improving EBN's nutritional.

4. Conclusions

This study has highlighted the potential benefits and trade-offs of enzymatic hydrolysis on EBN, focusing on both RUC EBN and by-product EBN. Enzymatic hydrolysis, utilizing bromelain, significantly enhanced the sialic acid content in EBN, with hydrolyzed samples (EBNch and EBNcph) showing higher levels compared to their non-hydrolyzed counterparts (EBNc and EBNcp). This suggests that enzymatic hydrolysis can effectively increase the nutritional value of EBN, particularly in terms of sialic acid, a key component known for its health benefits. However, the process also resulted in a reduction of antioxidant activity, as demonstrated by the lower DPPH radical scavenging activity in hydrolyzed samples. This trade-off indicates that while hydrolysis improves certain nutritional aspects, it may diminish others, necessitating a balanced approach when considering EBN for various applications in food and pharmaceuticals. Importantly, despite the reduction, antioxidant activity was retained to a considerable extent, allowing the promotion of hydrolysate as a nutritionally viable product. The study also confirmed the safety of EBN hydrolysates regarding heavy metal contamination. None of the samples, including byproduct and hydrolyzed EBN, contained detectable levels of arsenic, lead, mercury, cadmium, or antimony, aligning with regulatory standards and previous research findings. This highlights the potential of EBN, particularly hydrolyzed by-products, as safe and valuable ingredients in health-related products. Amino acid profiling further supported the nutritional advantages of hydrolysis. Hydrolyzed EBN (EBNch and EBNcph) exhibited significant enhancements in specific amino acids, notably leucine, which was absent in non-hydrolyzed samples. This enhancement could provide substantial benefits for muscle health and overall nutrition, especially for populations with higher protein requirements. Overall, the findings suggest that enzymatic hydrolysis of EBN, particularly when applied to by-products, can produce value-added products with enhanced sialic acid content and improved amino acid profiles, though with some reduction in antioxidant activity. In additional, the enzymatic hydrolysis process can be a better choice for the processing of heavy feather RUC EBN and by-product EBN, saving time in the picking step of the primary process, achieving higher product recovery for heavy feather RUC EBN, and producing valuable by-products from EBN. Future research should focus on optimizing hydrolysis conditions to maximize these benefits while mitigating the loss of antioxidant properties. Exploring different enzymes and processing methods may also provide insights into preserving or enhancing the antioxidant activity in hydrolyzed EBN, thereby broadening its application scope in the food and pharmaceutical industries.

5. Conflict of Interest

Authors have declared that no competing interests exist.

6. References

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