Bai *et al. Applied Biological Chemistry* (2023) 66:36
https://doi.org/10.1186/s13765-023-00794-2

KSABC
The Korean Society for Applied Biological Chemistry

ARTICLE

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The determination of epidermal growth factor in Edible bird's nest by enzyme-linked immunosorbent assay

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Abstract

Edible bird's nest (EBN) is a traditional food which was nourishing and functional. Particularly, there is the epidermal growth factor (EGF) in EBN, which is thought to play an important role in promoting skin repair. However, the type and content of EGF in EBN were not determined yet. In this study, the type of EGF in EBN was identified as bird EGF by enzyme-linked immunosorbent assay and this method was validated to be accurate and precise. Moreover, it was found that the content of EGF in raw-unclean EBN, raw-clean EBN and stewed EBN was 3000 pg/g–4000 pg/g and there were no significant differences, which suggested that the batches, origins, forms, stewing temperatures and stewing times of EBN had no effect on the content of EGF in EBN. However, it was due to that enzyme destroyed the primary structure of EGF, the EGF content of neutral protease and trypsin hydrolysates of EBN was lower than that of flavor enzymes, alkaline protease and pepsin hydrolysates of EGF. This study was the first to determine the type and content of EGF in EBN, and provided a theoretical basis for the selection and processing of EBN and using EBN as a source of EGF.

Keywords Edible bird's nest, Epidermal growth factor, Content

Introduction

Edible bird's nest (EBN) is an edible biological product made from the salivary gland secretions of swiftlets through picking and other processes. These swiftlets belong to the genera *Collocalia* and *Aerodramus*, and inhabit South-East Asia and neighboring islands. According to Chinese traditional medicine, EBN was beneficial to the lung, kidneys, spleen, and stomach [1]. On the other hand, modern research showed that EBN had the functions of improving neurodegenerative diseases [2, 3], anti-oxidation [4], anti-aging [5], anti-influenza [6], inhibiting hemagglutination [7], and immune regulation [8]. Moreover, it also could enhance intelligence and memory [9, 10], improve cardiovascular diseases [11], and promote cell proliferation [12]. Therefore, EBN was very popular in China. It was found that epidermal growth factor (EGF) might play an important role in some functions, such as promoting cell proliferation.

EGF is one of the many growth factors and is usually referred to human EGF if not specified specifically. EGF is a peptide composed of 53 amino acid molecules, which contains three disulfide bonds. The molecular weight of EGF is about 6000 Da, and the isoelectric point of EGF is 4.6 [13]. EGF participated in various physiological activities, such as cell division, proliferation, and migration [14–17]. It could promote the growth of epidermal cells, epithelial tissue regeneration, and wound healing, which had been widely used

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in the clinical treatment of skin wounds [18–20]. For example, oral administration of EGF could effectively inhibit intestinal inflammation in neonatal rats with necrotizing enterocolitis, and repair lipopolysaccharide-induced injury on rat small intestine crypt epithelial cells to improve the intestinal barrier function [21–23]. In addition, stimulation of glioblastoma cells with EGF could induce the upregulation of xCT and promote cell death under glucose deprivation [24]. Moreover, EGF, as a potential biomarker for the progression of chronic kidney disease, could exert protective effects on high glucose-induced podocytes injury via enhancing cell proliferation and inhibiting cell apoptosis [25].

For EGF in EBN, it could be divided into two types of concepts. First, a large number of peptides with functions similar to EGF (such as promoting skin repair) were found in EBN [26–29], which can be considered as the broad sense EGF of EBN, e.i. human-like EGF peptides. The second is the narrow sense EGF of EBN, which has a similar structure as other discovered EGFs. However, previous studies on EBN mainly focused on sialic acid. Interestingly, regarding the EGF in EBN in a narrow sense, there is no literature report to prove which EGF is contained in EBN, for example, whether there is EGF with a similar structure to rat EGF, mice EGF, human EGF, and bird EGF has not yet been elucidated. Moreover, it is also worth exploring whether processing conditions affect EGF content in EBN. This is an interesting and meaningful field.

Enzyme-linked immunosorbent assay (ELISA) is a technique in which known antigens or antibodies are adsorbed on the surface of a solid phase carrier, and the enzyme-labeled antigen and antibody react on the surface of the solid-phase carrier. This technique could determine macromolecular antigens and specific antibodies. So far, many kinds of EGF ELISA kits, such as rat EGF ELISA kit, mice EGF ELISA kit, human EGF ELISA kit and bird EGF ELISA kit, were successfully developed and were widely used for the determination of EGF content in other substances. Moreover, it was fast, sensitive, and easy to standardize the carrier. Therefore, ELISA is suitable for the determination of EGF in EBN.

Therefore, the purpose of this study is to use the existing EGF ELISA kits to detect the EGF content in EBN, so as to more accurately determine which type of EGF exists in EBN and how its content is. In addition, in order to direct the industry production, the effects of processing conditions on the EGF content of EBN were further explored. This study provided valuable information on the composition of EBN and contribute to further understanding of the health benefits of EBN. Moreover, the results of this study provided a scientific basis for using EBN as a source of EGF.

Materials and methods

Materials

The raw-unclean EBNs from different batches and places of origin were supplied by Technical Center, Xiamen Customs (Xiamen, China); The raw-clean EBNs, different forms of EBNs, and ready-to-eat EBNs (a kind of canned product) were obtained from Xiamen Yan Palace Seelong Food Co., Ltd. (Xiamen, China); 1×phosphate buffer saline (PBS) buffer and fetal bovine serum (FBS) were purchased from Sigma Aldrich (Shanghai, China); Rat EGF ELISA kit, mice EGF ELISA kit, human EGF ELISA kit, and bird EGF ELISA kit were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China); Alkaline protease (200,000 U/g), neutral protease (200,000 U/g), flavor enzymes (20,000 U/g), pepsin (3000 U/g) and pancreatin (4000 U/g) were obtained from Nanning Pangbo Biological Engineering Co., Ltd. (Nanning, China).

Pretreatment of EBN

Generally, according to the cleanliness of the EBNs, EBNs is divided into raw-unclean and raw-clean EBNs, and the difference is whether it contains natural feathers. The ready-to-eat EBN was prepared from raw-clean EBN through a series of processing. At present, most of the EBNs on the market were raw-clean EBN and ready-to-eat EBN. The raw-unclean or raw-clean EBN was milled by a mill (A11 basic S025, IKA, Staufen, Germany) and passed through a 100-mesh sieve, while the ready-to-eat EBN was homogenized using a homogenizer (T18DS25, IKA, Staufen, Germany).

Determination of EGF content in EBN

In this study, the raw-clean EBN was used to identify the EGF type in EBN. The type of EGF in EBN was identified by qualitative comparison with the EGF standards provided by the rat EGF ELISA kit, mice EGF ELISA kit, human EGF ELISA kit and bird EGF ELISA kit.

In detail, the raw-clean EBN (1.0 g) was dissolved in 9.0 mL PBS buffer, then the solution was homogenized and centrifuged at 10,000 rpm for 15 min to obtain the supernatant for determining EGF content. After the four kits were removed from the refrigerated environment, they were kept at room temperature for 60 min. If the ELISA plates were not used up after being opened, the remaining strips were stored in a sealed bag. Standard solutions with different concentrations (50 μL) were added to the bottom of the ELISA wells. For the samples, the sample diluent (40 μL) and 10 μL of EBN solution were added, then the ELISA plate was shaken slightly to

mix. Then, ELISA reagent (100 μL) was added to each well, and PBS buffer was used as a blank control without the sample and ELISA reagent. After the plate was sealed and incubated at 37 °C for 60 min, the liquid in the wells was discarded. The 20×washing solution was diluted 20 times with distilled water. Subsequently, 1×washing solution was added to each well until fill for standing 30 s, and then discarded. This process was repeated 5 times, and the remaining water in each well was drained at the last time. Next, chromogenic reagent A (50 μL) and chromogenic reagent B (50 μL) were added to each well in sequence. After mixing and reacting in the dark at 37 °C for 15 min, the reaction was terminated by 50 μL termination buffer. Meanwhile, the color of the reaction solution changed from blue to yellow immediately. Finally, the absorbance of the reaction solution was determined at 450 nm using a microplate reader (Epoch, Bio Tek, Vermont, USA). Importantly, the absorbance measurement should be conducted within 15 min after adding the termination buffer.

Comparison of exaction agent

To find an applicable exaction agent for EBN and its product, the raw-clean and ready-to-eat EBN (1.0 g) were dispersed in 9.0 mL PBS buffer, 0.2% FBS solution, 0.4% FBS solution, and saturated FBS solution, respectively. Then, the suspension was homogenized and centrifuged at 10,000 rpm for 15 min to obtain the supernatant for further determination of EGF content by bird EGF ELISA kit as described in section "Determination of EGF content in EBN".

EGF content in EBN from different batches, origins and forms

Nine batches of raw-unclean EBN from Indonesia, and raw-unclean EBN from Laba, East Kalimantan, Pariji, and South Kalimantan were selected. The bar-like EBN, triangular EBN, cup EBN, and EBN fragments were also collected for further determination. The EBN (1.0 g) was dispersed in 9.0 mL PBS buffer, then the suspension was homogenized and centrifuged at 10,000 rpm for 15 min to obtain the supernatant for determination of EGF content by bird EGF ELISA kit as described in section "Determination of EGF content in EBN".

EGF content in EBN treated by different stewing temperatures and times

The raw-clean EBN (1.2 g) was mixed with 5% hot syrup (45.0 g) in the glass bottle and the lid was closed tightly. Subsequently, the glass bottles were put into the sterilization pot to stew. For different stewing times, after rising to 121 °C in 5 min, the temperature was kept for 0, 10, 15, 20, 30, and 40 min, respectively. And for different stewing temperatures, after the temperature rose to 90, 95, 100, 115, 121 and 128 °C in 5 min, respectively, the temperature was kept for 15 min. When the pressure dropped to zero, the samples were taken out and placed in cold water to cool to room temperature slowly. The EGF content was determined by bird EGF ELISA kit as described in section "Determination of EGF content in EBN".

EGF content in EBN hydrolysates (EBN peptides)

The raw-clean EBN (2.0 g) was suspended in 98.0 g distilled water and then stewed in boiled water for 120 min. After the reaction, the suspension was cooled to room temperature and homogenized at high speed for 2 min. The pH of the suspension was adjusted to 7.0, 9.5, 8.0, 2.5, and 7.5, respectively, by 0.1 M HCl and 0.1 M Na_2CO_3 solution. Subsequently, flavor enzymes, alkaline protease, trypsin, pepsin, and neutral protease (600 U/g raw-clean EBN) were added into suspension, respectively, which reacted at 50 °C for 6 h and then inactivated in ice water for 10 min. Finally, the suspension was centrifuged at 10,000 rpm for 15 min to obtain the supernatant for the determination of EGF content by bird EGF ELISA kit as described in section "Determination of EGF content in EBN".

Statistical analysis

In this study, all experiments were performed three times and the results were expressed as mean with standard deviation (SD). The data were analyzed by SPSS software (Version 22.0) and one-way analysis of variance (ANOVA) was used. In addition, Duncan's test was used to analyze the differences among the mean value, and differences were considered as statistical significance if $p < 0.05$.

Results and discussion

Applicability of bird EGF ELISA kit

At present, because no EGF antigen in EBN has been found, there is no specific ELISA kit for EGF detection in EBN on the market. Therefore, four other EGF kits were used to determine the EGF content in raw-clean EBN, and PBS buffer was used as the negative control. As shown in Fig. 1, the concentration of the standard solution and absorbance were linearly related within a specified range in four kits. Moreover, all the minimum detectable concentrations described in the product manuals could be reproduced. It was suggested that the four kits used in this study were effective. The rat, mice, and human EGF ELISA kits were negative for all raw-clean EBN samples, that is, no rat, mice, or human EGF was detected in the EBN samples (Table 1). In contrast, the bird EGF ELISA kit determined the presence of EGF in EBN, which confirmed the presence of related

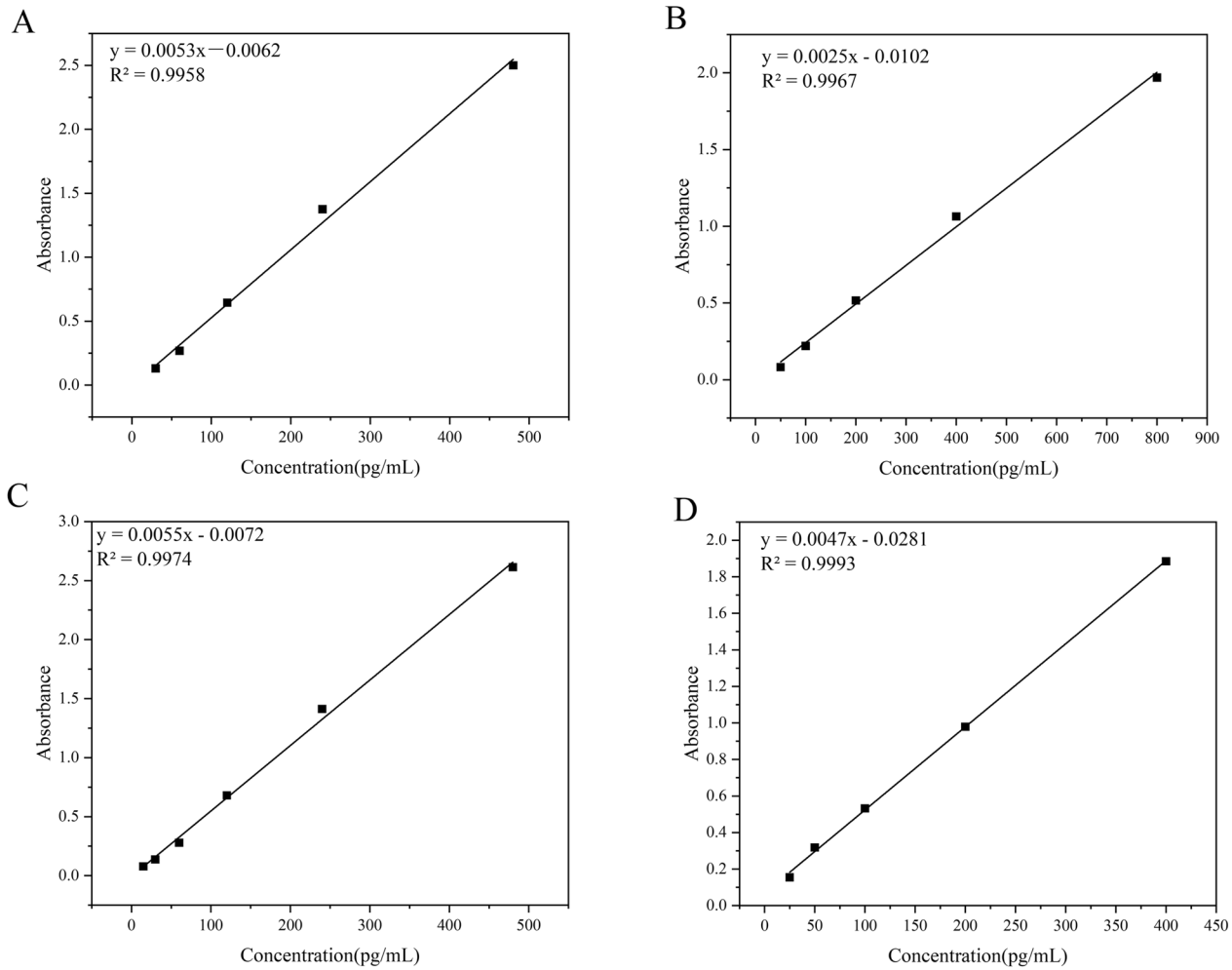

Fig. 1 Standard curves of rat (**A**), mice (**B**), human (**C**) and bird (**D**) EGF ELISA kit

Table 1 Detection results of four EGF ELISA kit

Samples	Rat EGF	Mice EGF	Human EGF	Bird EGF
Control (PBS buffer)	–	–	–	–
Raw-clean EBN	–	–	–	+

The "–" in the table meant that the EGF was not detected, and "+" meant that the EGF was detected

antigens. That is, the result proved that there was bird EGF in EBN, which is described as EGF in the following text. Therefore, the bird EGF ELISA kit was selected for further experiments. In fact, EBN comes from swiftlets, which belong to birds. Therefore, it is reasonable that the EGF ELISA kits of other birds can detect the corresponding antigens in EBN.

Effect of exaction agents on EGF content in EBN

EGF is a kind of water-soluble peptide. At present, most of the EBNs on the market were raw-clean EBNs and ready-to-eat EBNs. It was speculated that different processing techniques might result in difference content of EGF in EBN, but this has not been reported yet. Based on the above, the effects of extraction agents on EGF content in raw-clean EBN and ready-to-eat EBN were studied first. As shown in Fig. 2, when PBS buffer, 0.2% FBS, 0.4% FBS, and saturated FBS were used as extractants, the content of EGF was basically the same ($p > 0.05$). It was indicated that there was no significant effect of extraction agent on EGF content in EBN and its products. However, the spiked recovery rate of the method using PBS as the extraction solvent was higher than that of the other three solutions as the extraction solvent (Table 2). Therefore, PBS buffer was selected as the extraction solvent for sample pretreatment in this study

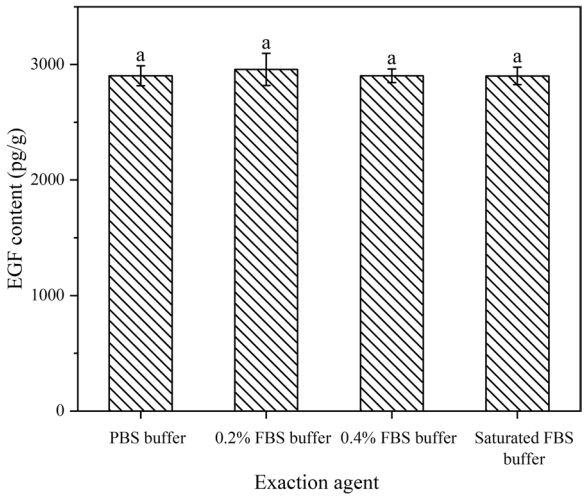

Fig. 2 Effect of exaction agent on EGF content in EBN

Stability of bird EGF ELISA kit

As shown in Fig. 3 and Table 3, the experiment was performed using standard ingredients in the bird EGF ELISA kit and a standard curve was drawn. The value of R^2 was 0.9995, which met the requirement that the kit linear coefficient was greater than 0.95. In this experiment, each test was repeated three times with six parallel replicates per time, and each parallel was repeated three times. The relative standard deviation (RSD) value of the three replicates ranged from 2.39 to 5.42%, meeting the requirement that the variation coefficient within the batch was less than 10%. The SD value of the three tests was 191.9 pg/g and the RSD value was 5.25%, which met the requirement of variation coefficient between batches less than 15%. Therefore, the bird EGF ELISA kit was suitable for the determination of EGF in EBN and its products.

Effect of batches on EGF content in raw-unclean EBN

After determining the applicability of the assay, the effect of the batches on the EGF content in raw-unclean EBN was first investigated. It was due to that the quality of EBN from different batches might be affected by the status of swiftlets. It was found that there was the same content of EGF in nine batches of raw-unclean EBN from Indonesia (Fig. 4A, $p > 0.05$). It was suggested that using the detection method described in this study, there was no significant difference in EGF content in different batches of EBN from the same origin. That is, the EGF content in EBN from the same origin had good stability.

Table 2 Recovery of different extraction solvents (%)

Samples	PBS buffer	0.2% FBS buffer	0.4% FBS buffer	Saturated FBS buffer
Raw-clean EBN	84.53 ± 1.56^{d}	64.41 ± 1.22^{c}	45.57 ± 1.03^{b}	24.15 ± 2.01^{a}
Ready-to-eat EBN	89.42 ± 0.75^{d}	71.23 ± 1.86^{c}	49.28 ± 0.48^{b}	31.58 ± 1.90^{a}

Each value in the table was the mean ± standard deviation of three replicates. Values in the same row with different letters were significantly different at $p < 0.05$, based on Duncan's test

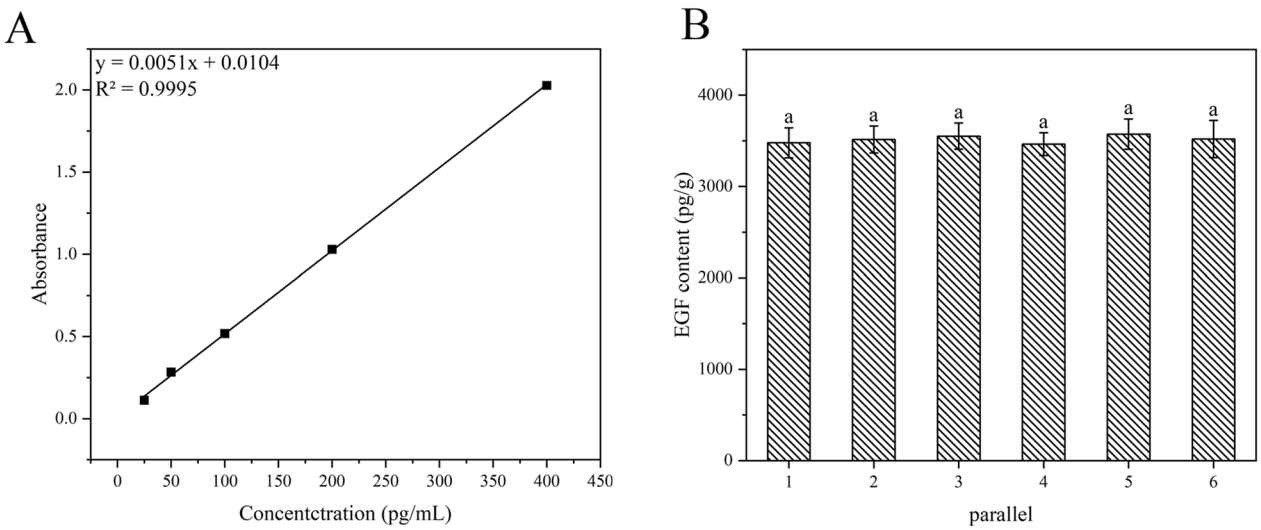

Fig. 3 Standard curve (**A**) and parallel and repeatability tests (**B**) of bird EGF ELISA kit

Table 3 Parallel and repeatability tests of bird EGF ELISA kit

Test	Parallel	$\overline{X}$ (pg/g)	RSD vaules	Total mean (pg/g)	Total RSD vaules
First	1	3742.3 ± 76.9	5.45%	3658.4 ± 191.9	5.25%
	2	3652.3 ± 67.5			
	3	3343.9 ± 75.4			
	4	3678.9 ± 146.1			
	5	3657.7 ± 61.2			
	6	3414.1 ± 95.3			
Second	1	3570.8 ± 45.6	2.39%		
	2	3575.5 ± 71.8			
	3	3550.5 ± 21.3			
	4	3432.4 ± 97.2			
	5	3603.8 ± 124.5			
	6	3550.5 ± 21.3			
Third	1	3793.5 ± 134.6	4.36%		
	2	3849.8 ± 66.9			
	3	3899.6 ± 33.3			
	4	3922.2 ± 86.2			
	5	3897.9 ± 102.8			
	6	3854.9 ± 67.3			

Each value in the table was the mean ± standard deviation of three replicates

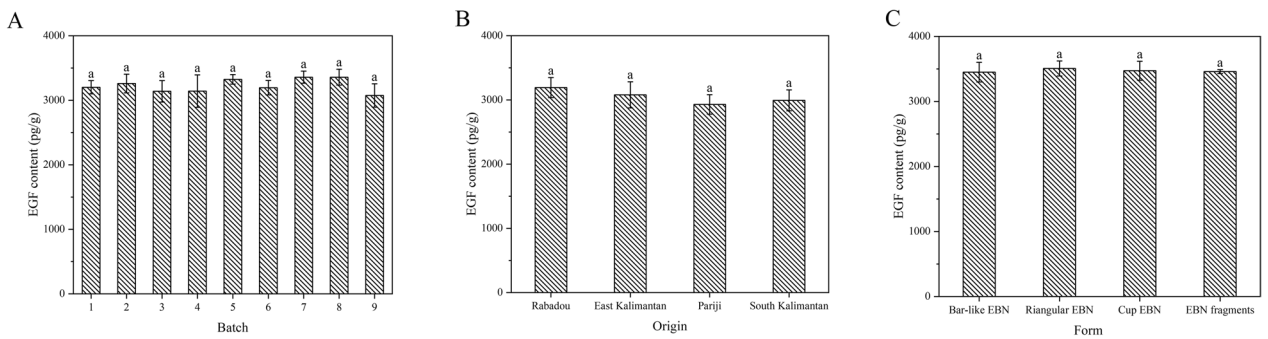

Fig. 4 The EGF content in EBN from different batches (**A**), origins (**B**) and forms (**C**)

Effect of origins on EGF content in raw-clean EBN

Subsequently, the content of EGF in raw-clean EBN from different origins was determined. As shown in Fig. 4B, the EGF content of raw-clean EBN from Rabadou, East Kalimantan, Pariji and South Kalimantan ranged from 3000 to 3200 pg/g. However, there was no significant difference in the EGF content of raw-clean EBN from the four origins above ($p > 0.05$). Swiftlets have high requirements on habitat. The island countries in Southeast Asia have similar climates and a wide variety of plants and animals, which are suitable for the survival of swiftlets. Therefore, EBN produced in Southeast Asia has high-quality and stable EGF content.

Effect of forms on EGF content in raw-clean EBN

Generally, the EBN was divided into bar-like EBN, triangular EBN, and cup EBN. In the transportation and processing, the EBN might be broken to varying degrees, and the fragments were called EBN fragments. In fact, different forms of EGF were used in different products according to requirement. In this section, the effect of EBN form on EGF content in EBN was investigated. As shown in Fig. 4C, the EGF content of bar-like EBN, triangular EBN, cup EBN, and EBN fragments ranged from 3300 to 3500 pg/g, which had no significant difference ($p > 0.05$). As expected, the content of EGF was not related to the form of EBN. It is well known that the primary structure of the peptide will not be easily changed by low-energy

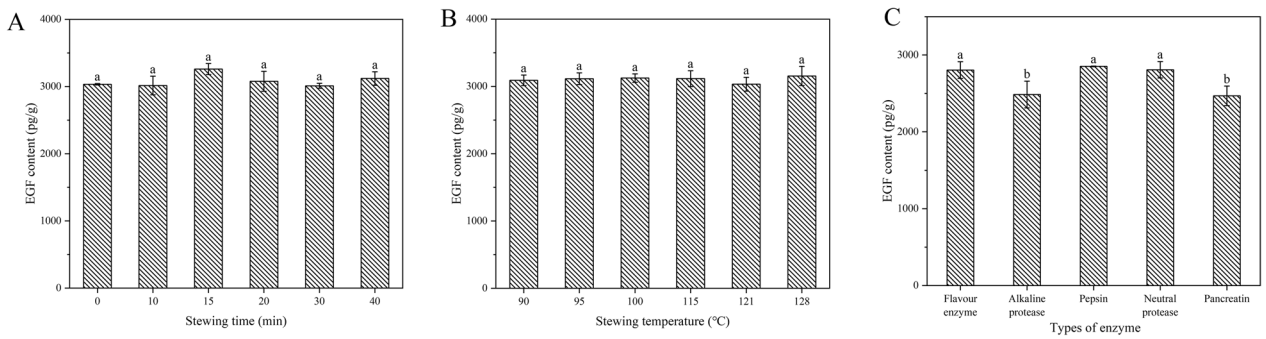

Fig. 5 The effects of stewing time (**A**), stewing temperature (**B**) and enzymatic hydrolysis (**C**) on EGF content in EBN

input processing methods such as knife cutting and crushing [30]. As a kind of peptide, EGF also has a stable structure. Therefore, the result is reasonable.

Effect of stewing times on EGF content in EBN

At present, the ready-to-eat EBN products processed by factories are more and more popular among consumers. In the processing of ready-to-eat EBN products, the most important step is stewing. Therefore, in this section, the effects of different stewing times on the EGF content in ready-to-eat EBN products were investigated. The result was shown in Fig. 5A. At the temperature of 121 °C set in this section, the content of EGF in EBN ranged from 3000 to 3200 pg/g, which did not change with the increase of stewing time. This result indicated that stewing time had no effect on the content of EGF in EBN. As mentioned above, as a peptide, EGF has a relatively stable primary structure and is not easily destroyed by stewing.

Effect of stewing temperatures on EGF content in EBN

The stew temperature can affect the taste and state of ready-to-eat EBN, which is an important parameter in the processing. Therefore, continuing from the above, in this section, the effects of different stewing temperatures on the EGF content in ready-to-eat EBN products were investigated. At the stewing temperature of 90 to 128 °C, the content of EGF in EBN ranged from 3000 to 3500 pg/g, and there was no significant difference (Fig. 5B, $p > 0.05$). It was suggested that stewing temperature could not affect the content of EGF in EBN, which might be due to the thermal stability of EGF as stated above.

Effect of enzymatic hydrolysis on EGF content in EBN

Compared with proteins, peptides are more conducive to human absorption and have stronger antioxidant activity. Moreover, more and more researchers have found that the hydrolysates of EBN (EBN peptides) have functions similar to EGF [25]. EBN peptides are expected to become a new generation of tonic food. Therefore, in this section, the EGF content in EBN hydrolysates hydrolyzed by different enzymes was investigated. As shown in Fig. 5C, the content of EGF in EBN hydrolysates (EBN peptides) hydrolyzed by flavor enzymes, alkaline protease, pepsin, neutral protease and pancreatin was 2804, 2851, 2807, 2486, and 2468 pg/g, respectively. In particular, the EGF contents of EBN hydrolysates (EBN peptides) hydrolyzed by neutral protease and pancreatin were significantly lower than that of the other three hydrolysates (EBN peptides) ($p < 0.05$). This result indicated that the different enzymatic hydrolysis had a significant effect on the EGF content of EBN. It might be because some enzyme cleavage reaction destroyed the primary structure of EGF, and different enzymes acted on different sites, resulting in different degrees of EGF destruction.

Conclusion

In summary, rat, mice, human, and bird EGF ELISA kits were used to determine the content of EGF in EBN in thisstudy. It was found that there was bird EGF in EBN but no rat, mouse, or human EGF. Moreover, the bird EGFELISA kit was suitable for the determination of EGF in EBN, and the standard curve linearity and variationcoefficient met the requirements. In addition, different batches, origins, and forms of EBN and stewing time andtemperature in EBN processing did not affect the content of EGF in EBN, which confirmed that EGF in EBN hadgood stability, including thermal stability. However, it was found that the different enzymatic hydrolysis hadsignificant effects on the EGF content of EBN, neutral protease and pancreatin could destroy the primary structureof EGF in EBN. In short, the above results proved the existence of EGF in EBN through scientific methods, whichprovided good support for the value display of EBN. However, there are still some shortcomings in this study. Forexample, the bird EGF ELISA kit used in this study can only measure one type of EGF with a

specific structure,whether more kinds of EGF are present in EBN has not been elucidated. Furthermore, there were many othersubstances in EBN have been reported to have similar effects to EGF, such as sialic acid. It has not been definedwhich of these substances belong to the EGF class. Therefore, these fields still needed to be further explored.

Abbreviations

EBN	Edible bird's nest
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate buffer saline
FBS	Fetal bovine serum

Acknowledgements
Not applicable.

Author contributions
Conceptualization, WB, and BG; methodology, WB, FD, XZ, YH and YX; software, WB; validation, WB, FD, XZ, and NW; formal analysis, BW, FD and BG; investigation, BW and QF; resources, QF; data curation, WB, XL and BG; writing—original draft preparation, WB and FD; writing—review and editing, XL and BG; visualization, XL and BG; supervision, XL and BG; project administration, QF; funding acquisition, QF. All authors have read and agreed to the published version of the manuscript.

Funding
This research was funded by Xiamen Science and Technology Plan Project (3502Z20224024).

Availability of data and materials
The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human and animal subjects.

Competing interests
The authors declare that they have no competing interests.

Received: 7 March 2023 Accepted: 30 May 2023
Published online: 09 June 2023

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