Hindawi
Journal of Food Biochemistry
Volume 2023, Article ID 3211692, 10 pages
https://doi.org/10.1155/2023/3211692

Research Article

Fractionation of Simulated Digestive Products of Bird's Nest *In Vitro*: Skin Whitening Activity of Fractions

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Received 3 April 2023; Revised 7 June 2023; Accepted 18 August 2023; Published 25 August 2023

Academic Editor: Rotimi Aluko

Edible bird's nest (EBN) has been proven to exhibit whitening activity. In spite of this, the relationship between the digestive products of EBN and the whitening activity is still unclear. Hence, the B16-F10 cell model was used in this study to study the whitening activity of simulated EBN digestion products *in vitro*. The simulated digestive juices showed significant inhibition of tyrosinase (TYR) ($P < 0.05$), and the release rate of free sialic acid was 21.87% in EBN digestive juices. After simulated gastrointestinal digestion, the relative contents of <1 kDa components, 1–3 kDa components, and >3 kDa components were 42.63%, 15.05%, and 42.32%, respectively. EBN digestive products were further fractionated, and the fraction of <1 kDa (EPP-1) exhibited the highest tyrosinase inhibitory activity with 83.12% and its peptides content was 92.44%. The EPP-1 significantly inhibited tyrosinase activity and decreased melanin content ($P > 0.05$) by regulating the cAMP/MITF/TRP-1/TRP-2 tyrosinase signaling pathways in the B16-F10 cell experiment. Based on these findings, our data suggest that the digestive products of EBN may potentially be fit for developing a new generation of natural whitening health products.

1. Introduction

Edible bird's nest (EBN) is a nest made by mixing saliva and feature of swiftlets, which has been proven to have whitening, antioxidant, anti-inflammatory, immune regulation, antiaging, and other biological activities [1]. EBN is mainly composed of proteins and carbohydrates, and proteins account for more than 50% of the dry weight of EBN on average, which is a key factor in the nutritional and medicinal functions of EBN [2–4]. It has been reported that hydrolyzed EBN showed higher free radical scavenging abilities [5, 6], antimicrobial property, and anti-hyperglycemic property [7]. Traditionally, people stew EBN for several hours before eating, but the majority of the proteins are still undissolved and sialic acid is in the bound form, and it is hard for humans to absorb it [8].

The digestion of EBN *in vitro* is an efficient method to release its bioactive ingredients and improve its functional properties. It has been reported that digested EBN under simulated gastric fluid released smaller peptides and free sialic acid exhibited better whitening properties compared to undigested EBN [8]. Zack discovered that intracellular tyrosinase activity and melanin formation were both significantly inhibited by the simulated digestion products of EBN *in vitro* [8]. Sialic acid and active peptides from EBN are the main whitening active ingredients in EBN digestive products [9]. Chan et al. revealed that sialic acid, the primary element in EBN skin-whitening effect, could significantly suppress tyrosinase activity and diminish melanin synthesis in B16 murine melanoma cells and A375 human melanoma cells [10]. Ghassem et al. found two novel antioxidant peptides isolated from EBN protein hydrolysates, which exhibited whitening activity by protecting cells from oxidative damage and scavenging oxygen-free radicals [11]. Fan et al. found that simulated gastrointestinal-digestion products of EBN are fit for the development of a fresh generation of whitening health product [12].

EBN is known for its proven skin-whitening properties. Protein and sialic acid are the two most important whitening ingredients in EBN [13]. However, there is a lack of detailed

reports on the composition and content changes of sialic acid and protein hydrolysates during simulated digestion of EBN, and the relationship between digestive product composition and whitening activity needs to be further studied. In this work, the relationship between the composition changes of digestive products of EBN during the *in vitro* simulated digestion process and the whitening activity was studied. Digested products of EBN were further fractionated based on the molecular weight, and whitening activity of fractions after fractionation was studied by the B16 murine melanoma cells model. This study is beneficial for the preparation of EBN-whitening active ingredients by enzymatic hydrolysis.

2. Materials and Methods

2.1. Materials and Reagents. Malaysia bird's powder was purchased from Qingdao Zhengdian Biotechnology Co., Ltd. (Shandong, China). RMPI basal medium, fetal bovine serum, Dulbecco's phosphate buffer, and 0.25% pancreatin (with EDTA) were purchased from Procell Life Science & Technology Co., Ltd. (Hubei, China). Alpha-arbutin was purchased from Shanghai Macklin Technology Co., Ltd. (Shanghai, China). Pepsin, pancreatin, and sialic acid standard were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Tyrosinase was purchased from Hefei Bomei Biotechnology Co., Ltd. (Anhui, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide was purchased from Sigma-Aldrich Trading (Shanghai) Co., Ltd. (Shanghai, China). The Reverse Transcription Kit, SYBR ® Premix Ex Taq TM, and RNA extraction kit were purchased from TransGen Biotech Ltd. (Beijing, China). All other chemicals and reagents used in this work were analytically pure, while the reagents for high-performance liquid chromatography (HPLC) analysis were chromatographic pure.

2.2. Simulated Gastrointestinal Digestion of EBN In Vitro. As mentioned earlier [14, 15], EBN was simulated for gastrointestinal digestion *in vitro* with slight modifications. The EBN solution was digested *in vitro* by a two-step digestion process to simulate gastrointestinal digestion. For this process, EBN powder (1.00 g) was dissolved in 50 mL saline. The solution pH was modified to 2.0 with 0.1 M HCl. Then, pepsin (0.02 g) was added to the mixed solution, and the mixture was digested at 37°C for exactly 4 h. Samples were taken at 0, 0.5, 1, 2, 3, and 4 h during gastric phase simulation digestion. The inhibitory activity of samples from each time point against tyrosinase was measured as described below. Finally, the pH of solution was adjusted to neutral to stop the digestion.

After 2 h of gastric digestion, the resultant peptic digest was further digested by the addition of pancreatin (0.02 g); then, the intestinal digestion solution pH was adjusted to 7.0, and the solution was protected from light and incubated at 37°C for 5 h. Samples were collected at 0, 1, 2, 3, 4, and 5 h during intestinal digestion, respectively. After the intestinal digestion, the solution was heated up to 90°C for 10 min to inactivate enzyme. The digests were centrifuged, and the supernatants were collected. The supernatants were vacuum dried for subsequent analysis. The inhibitory activity of samples from each time point against tyrosinase was measured as described below.

Tyrosinase inhibition was assayed as described by Wang et al. [16] with minor changes. First, L-tyrosine (0.5 mg/mL, 200 μL, dissolved in phosphate buffer, pH 6.8) was added to the tube that contained the simulated digestive products of EBN (5 mg/mL, 150 μL, dissolved in PBS, pH 6.8) at 37°C for 10 min. Next, tyrosinase (500 U/mL, 50 μL) was mixed with the system to start the reaction. The absorbance at 475 nm of the mixture was measured after incubating at 37°C for 15 min. Then, the tyrosinase inhibition rate was evaluated, as given in the following equation:

$$\text{tyrosinase inhibition rate}\,(\%) = \frac{(A_1 - A_2) - (B_1 - B_2)}{A_1 - A_2} \times 100, \quad (1)$$

where A_1 was the absorbance without the digestive products of EBN, A_2 was the absorbance with the PBS as the control, B_1 was the absorbance of EBN digestive products with the tyrosinase, and B_2 was the absorbance with the digestive products of EBN but without the tyrosinase.

2.3. Molecular Weight Distribution. The molecular weight distribution of simulated digestion products of EBN *in vitro* was measured by gel permeation chromatography using a high-performance liquid chromatography (HPLC) system equipped with a TSK gel analytical column (G2500 PWXL, Tosoh, Japan). The cytochrome c (12500 Da), aprotinin (6500 Da), bacitracin (1450 Da), Gly-Gly-Tyr-Arg (451 Da), and Gly-Gly-Gly (189 Da) are treated as the peptide standards. The following conditions were applied: the sample concentration of 2.0 mg/mL, sample volume of 10 μL, a mobile phase of trifluoroacetic acid/acetonitrile/water (0.1 : 45 : 55, v/v/v), a flow speed of 0.5 mL/min, and the UV wavelength of detection of 220 nm.

2.4. Fractionation of Simulated Digestive Products of EBN In Vitro. The simulated digestive products of EBN were fractionated using ultrafiltration membranes of 3 kDa and 1 kDa, and the fractions of >3 kDa, 1–3 kDa, and <1 kDa were obtained by vacuum freeze-drying, and the fraction of <1 kDa was selected for subsequent cellular experiments and named as EPP-1.

2.5. Determination of Basic Components of EPP-1

2.5.1. Total Sugar Content. The total sugar was measured by the phenol-sulfuric acid technique as described by Zhang et al. [17].

2.5.2. Determination of Sialic Acid Content. Then, the content of sialic acid was determined as described by Alwael et al. [18] with slight modifications. Sample (50 mg) was mixed with 1% aqueous solution of phosphoric acid (40 mL)

in a conical flask (100 mL) in hydrolysis at 100°C waterbath for 20 min. The solution was allowed to cool at indoor temperature and then centrifugated at 5000 rpm for 10 min. The supernatant (1 mL) was mixed with 20 g/L solutions of 1,2-benzenediamine and hydrochloride in a shaking waterbath at 80°C for 40 min. The solution was cooled at room temperature and then filtered through a 0.22 μm polytetrafluoroethylene filter for further analysis.

2.5.3. Peptides Content. The content of peptides was measured by a biuret assay. Sample (200 μL, 2 mg/mL) was mixed with biuret reagent (400 μL) in the test tube. The absorbance at 540 nm of the mixture was measured after incubating for 15 min.

2.6. Cell Cytotoxicity of EPP-1. This experiment used the B16-F10 melanoma cells which were purchased from Procell Life Science & Technology Co., Ltd. (Hubei China). The MTT method was used to determine cell proliferation rate [19]. Mouse melanocytes cultured more than 3 times in log phase were selected, and the cell concentration was adjusted to 1×10^5 cells/mL after pancreatin digestion. Also, B16-F10 melanoma cells were seeded on a 96-well plate. The cells were cultured in 37°C and 5% CO_2 in an incubator for 12 h to enable them to fully adhere to the wall. After 12 h, various doses of EPP-1 were used to treat the cells. Also, after 24 h, the cytotoxic effects of EPP-1 on cells were assessed by using the MTT experiment. A microplate reader was used to read the absorbance at 490 nm, and the proliferation rate was evaluated as follows:

$$\text{cell viability } (\%) = \frac{A_2}{A_1} \times 100, \tag{2}$$

where A_1 was the blank absorbance and A_2 was the sample absorbance.

2.7. Cellular Tyrosinase Activity Assay. The rate of dopaquinone formation was used to estimate the intracellular tyrosinase activity [12, 20]. The measurement method was performed as previously described [21] with slight modifications. Cells were collected by digesting with pancreatin and centrifugation. DPBS was used to adjust cell concentration to 1×10^7 cells/mL. The supernatant was gathered after lysate centrifugation, and the protein content was measured. The supernatant of protein (0.2 mg/mL, 20 μL) was combined with L-dopa (1.0 mg/mL, 100 μL) for 30 min at 37°C. The solution was then tested at 475 nm, and then, the tyrosinase inhibition was calculated, as given in the following equation:

$$\text{tyrosinase inhibitory rate } (\%) = \frac{A_2}{A_1} \times 100, \tag{3}$$

where A_1 was the blank absorbance and A_2 was the EPP-1 absorbance.

2.8. Melanin Content Assay. The intracellular melanin content was measured with slightly modification according to the previous method [22]. After treating B16-F10 murine melanocytes with various doses of EPP-1 for 24 h, the cells were released by digestion using 0.25% pancreatin. Subsequently, the pellets were collected and dissolved in 1 M NaOH (containing 10% DMSO) and incubated in a constant temperature incubator at 80°C for 1 h. The cellular melanin contents were calculated by determining the absorbance at 405 nm as follows:

$$\text{relative melanin content } (\%) = \frac{(A_2/N_2)}{(A_1/N_1)} \times 100, \tag{4}$$

where A_1 was the blank absorbance, A_2 was the EPP-1 absorbance, N_1 was the number of cells in the blank group, and N_2 was the number of cells in the sample group.

2.9. Determination of Intracellular cAMP Content. For measurements of cellular cAMP content following incubations with EPP-1, after treating B16-F10 murine melanocytes with EPP-1 for 24 h, the culture medium was discarded and the cells were cleaned with DPBS. Then, cell lysates were prepared with RIPA lysis solution, and the intracellular cAMP content was determined with the cAMP ELISA Kit based on the instructions of the manufacturer.

2.10. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qPCR). Through real-time PCR analysis, the amounts of MITF, TYR, TRP-1, and TRP-2 mRNAs were quantified. To measure mRNA levels of melanin-related genes, mouse B16-F10 melanoma cells were treated with EPP-1 (1.0 mg/mL) and cultured for 24 h. The total RNA was extracted by the TRIzol-centrifuge column method [23]. Following the kit's directions, the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit were used to synthesize the first strand of cDNA using the isolated RNA as a template. The relative expression of melanin-related genes was detected by real-time fluorescence quantitative PCR. GADPH was applied as the reference gene. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the levels of relative mRNA. The primers used for qRT-PCR analysis are listed in Table 1.

2.11. Determination of Protein Expression Levels of TYR, TRP-1, and TRP-2. B16-F10 murine melanocytes were treated with EPP-1 (1.0 mg/mL) for 24 h, the culture medium was removed, and the cells were cleaned with DPBS. Then, cell lysates were prepared with RIPA lysis solution, and the intracellular expression of the melanin-related proteins TYR, TRP-1, and TRP-2 were determined with the ELISA kit according to manufacturer's instructions.

2.12. Statistical Analysis. All the experiments were carried out at least in triplicate independent determinations. The results were shown as the average ± standard deviation (SD) of the different independent experiments. The one-way ANOVA and Duncan's multiple range tests in SPSS statistics 26 were used to determine group differences.

Table 1: Primers used for real-time PCR.

Primer name	Direction	Nucleotide sequence (5′~3′)
MITF	Forward	GTGAATCGGATCATCAAGCAAG
	Reverse	GAGATCCAGAGTTGTCGTACAT
TYR	Forward	TTTCAACTGCGGAAACTGTAAG
	Reverse	ATTGTTCATTTGGCCATAGGTG
TRP-1	Forward	GATCCAGAAGCAACTTCGATTC
	Reverse	TACAAAGTGTTCCCAGGGTATC
TRP-2	Forward	GAGTATCCATCCAGACTACGTG
	Reverse	CGTCCTGGACCTAATAATGTGT
GAPDH	Forward	GGCAAATTCAACGGCACAGTCAAG
	Reverse	TCGCTCCTGGAAGATGGTGATGG

GraphPad Prism 8.0.2 was used to graph creation. The P value <0.05 was considered statistically significant.

3. Results

3.1. Inhibitory Effect of Simulated Gastrointestinal Digestion Products on Tyrosinase. Tyrosinase inhibition was measured to assess the whitening activity of digested EBN during the simulation of gastrointestinal digestion *in vitro*. As shown in Figure 1(a), the tyrosinase inhibition of the digested EBN sample group was always significantly higher than the control group during the gastric digestion phase ($P < 0.05$). The tyrosinase inhibition of the sample group was 27.18% at 1.0 h of gastric digestion. The tyrosinase inhibiting activity of digested EBN remained relatively stable after 2.0 h of gastric digestion ($P > 0.05$). Then, intestinal digestion was taken after 2 hours of gastric digestion.

As shown in Figure 1(b), compared with the gastric digestion stage, the tyrosinase inhibiting activity of digested EBN in the intestinal digestion stage significantly increased ($P < 0.05$). The tyrosinase inhibition rate of digested EBN remained relatively stable after 3.0 h of intestinal digestion ($P > 0.05$).

3.2. Analysis of Free Salivary Acid Content of EBN during Simulated Gastrointestinal Digestion Stage. As shown in Figure 1(c), the content of free salivary acid was continuously increased during gastric digestion and the free salivary acid reached 14.84% (16.75 μg/mL), following 2 h of digestion in the gastric region. During the digestive phase of the intestinal, the release rate of salivary acid reached 21.87% at 5 h. After 5 h, the rate leveled off and the change in free sialic acid content was not significant with the change in intestinal digestion time ($P > 0.05$).

3.3. Molecular Weight Distribution. The molecular weight distribution of digested EBN products during simulated gastrointestinal digestion was determined. As indicated in Figure 1(d), at the early stage of digestion, the EBN solution was largely composed of macromolecular glycoproteins, and the relative concentration of protein was more than 95%. At 1 h of gastric digestion, the peptides of various molecular weights were hydrolyzed from the EBN water-soluble proteins. Following 2 h of gastric digestion, the concentration of <1 kDa, 1–3 kDa, and >3 kDa was 25%, 17%, and 58%, respectively, and also following arrival at the intestinal digestion stage, a change in the pH of the digestive juice from acidic to neutral. The distribution of peptides changed rapidly after 1 h of digestion in the intestine. The content of <1 kDa components and 1–3 kDa components increased by 11.8% and 6.8%, respectively, and the >3 kDa components decreased by 18.7%. After 3 h of intestinal digestion, the concentration of <1 kDa components, 1–3 kDa components, and >3 kDa components were 42.63%, 15.05%, and 42.32%, respectively.

3.4. Effect of Molecular Weight on Tyrosinase Inhibitory Activity of Digested EBN. To determine the whitening active ingredients, the products of EBN digestion were further fractionated according to molecular weight. The tyrosinase inhibition rate was used to evaluate the whitening activity of fractionated components. It is shown in Figure 2 that the <1 kDa fraction of EBN peptides had a high tyrosinase inhibition rate of 83.12%, which was significantly high than the 1–3 kDa and >3 kDa components ($P < 0.05$). This may be related to the molecular weight size and the composition structure of the peptides in EBN-fractionated components. Finally, the <1 kDa component of EBN digestive products was selected for subsequent experiments, and it was named EPP-1.

3.5. Analysis of Basic Components of EPP-1. As shown in Figure 2, the tyrosinase inhibition rate of EPP-1 was the highest in EBN digestion products. Therefore, the components of EPP-1 were determined, as indicated in Table 2, of which the main component was the peptides at 92.44%, followed by the polysaccharide at 0.48% and sialic acid at 0.35%.

3.6. Effects of EPP-1 Treatment on B16-F10 Cell Viability. The safe concentration of EPP-1 on B16-F10 cells was measured by the MTT method, the results are shown in Figure 3(a). The cell viability was more than 90% when the concentration of EPP-1 was below 8 mg/mL and was nontoxic to B16-F10 cells. This range was a secure concentration range for the inhibition of intracellular tyrosinase activity and synthesis of melanin content. Arbutin at a content of 0.4 mg/mL was found to have a significant inhibitory effect on cell growth, with cell activity dropping below 90%. Therefore, the concentration of arbutin in the positive groups of the follow-up experiments was chosen as 0.1 mg/mL.

3.7. Effect of EPP-1 on Intracellular Tyrosinase Inhibition and Melanin Content. To study the whitening activity of EPP-1, the intracellular tyrosinase activity and melanin content experiments were used to evaluate the whitening activity. Arbutin was used as the positive control. The results are shown in Figure 3(b); compared with blank control, the tyrosinase activity was significantly inhibited by EPP-1 of

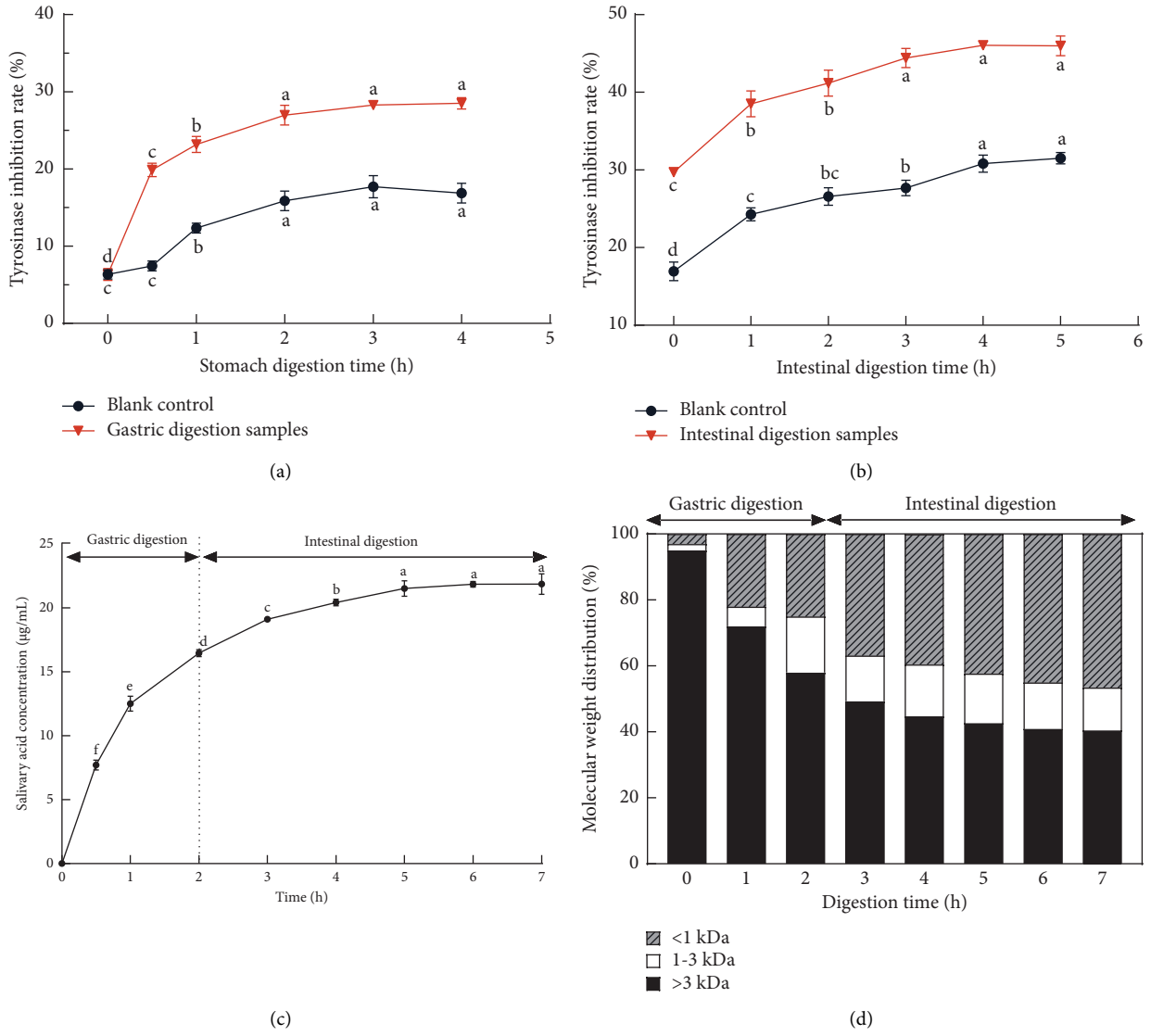

Figure 1: Whitening activity and physicochemical properties analysis of digested EBN during gastrointestinal digestion. (a) Tyrosinase inhibition of products with different gastric digestion times; (b) tyrosinase inhibition of products with different intestinal digestion times; (c) salivary acid level during gastrointestinal digestion; (d) molecular weight distribution of EBN digestive juices. Values with different letters are significantly different between the same curves ($P < 0.05$).

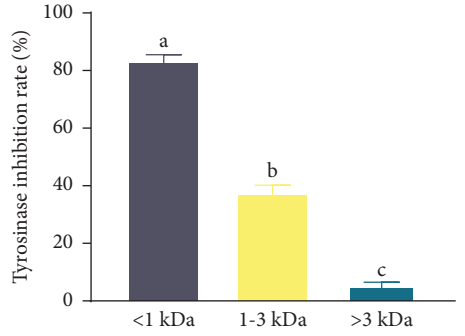

Figure 2: Effect of molecular weights on the tyrosinase inhibition rate. Values with different letters are significantly different between different groups ($P < 0.05$).

Table 2: Basic components of EPP-1.

Components	Peptides content	Total sugar content	Sialic acid
Content (%)	92.44 ± 0.03	0.48 ± 0.06	0.35 ± 0.02

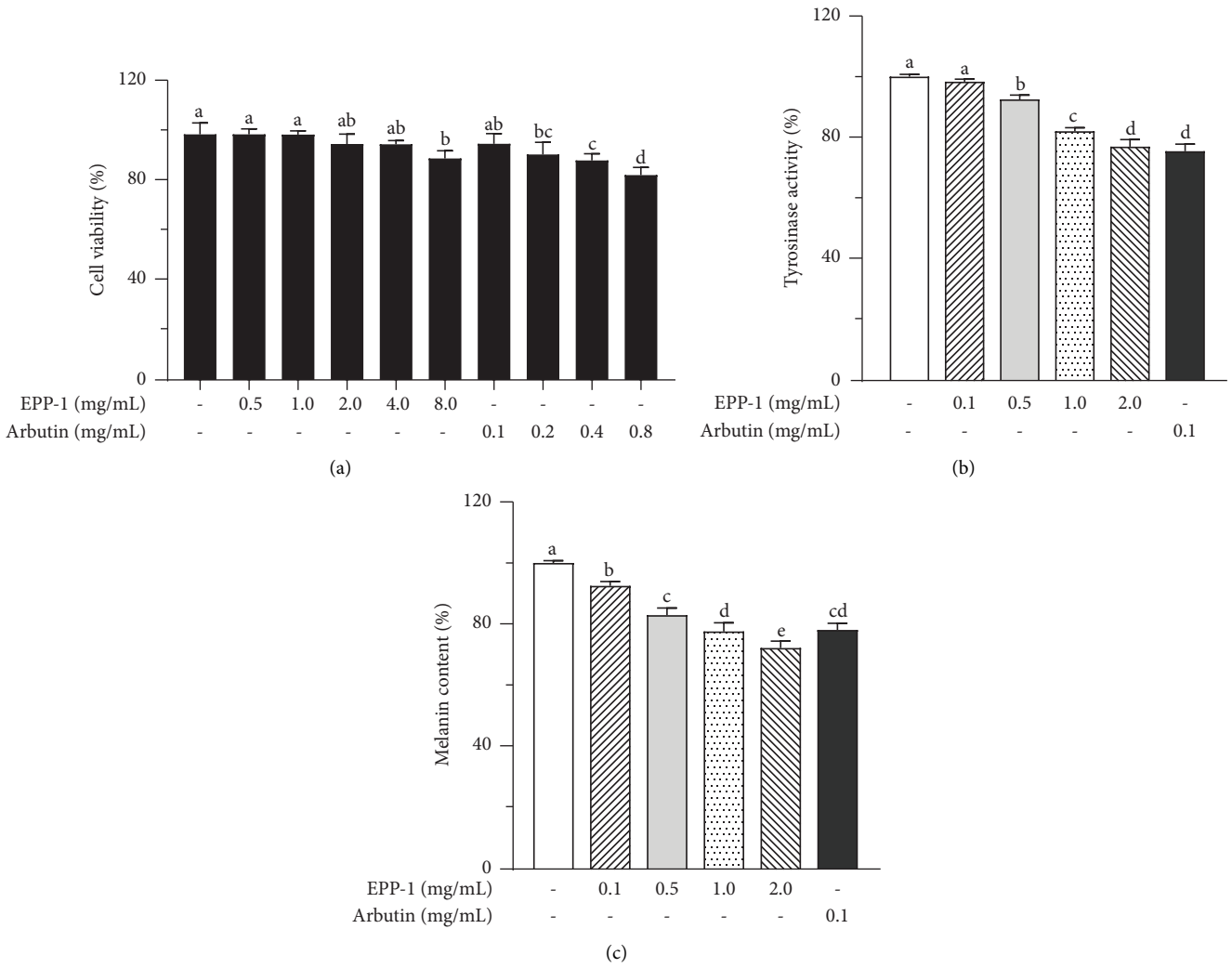

Figure 3: Cell viability of B16-F10 cells and effects of EPP-1 on tyrosinase activity and melanin content. The effect of EPP-1 on (a) cell viability, (b) tyrosinase activity, and (c) melanin content. Values with different letters within the same index are significantly different between columns ($P < 0.05$).

B16-F10 cells in the concentration range from 0.5 to 2.0 mg/mL ($P < 0.05$). The inhibition of EPP-1 (2.0 mg/mL) on tyrosinase at 22.66% was not significantly different from that of arbutin (0.1 mg/mL) at 24.13%. As shown in Figure 3(c), after treatment with different concentrations of EPP-1, intracellular melanin content was significantly decreased, compared to those in the blank group ($P < 0.05$). The inhibition of intracellular melanin content was 27.43% at the EPP-1 content of 2.0 mg/mL, and the inhibition of melanin synthesis was significantly better than in the positive group with arbutin (0.1 mg/mL) at 21.46% ($P < 0.05$).

3.8. Effect of EPP-1 on the Expression Levels of Melanin-Related Genes in B16-F10 Melanoma Cells. To further explore the whitening mechanism of EPP-1, intracellular cAMP content was determined. We found that the content of cAMP was significantly decreased in the arbutin and EPP-1 treated groups compared with those in the blank control group ($P < 0.05$) (Figure 4(a)). Moreover, the intracellular cAMP content in mouse B16-F10 melanoma cells was reduced by 28.78% and 36.94% when treated with 1.0 mg/mL and 0.1 mg/mL of EPP-1 and arbutin, respectively.

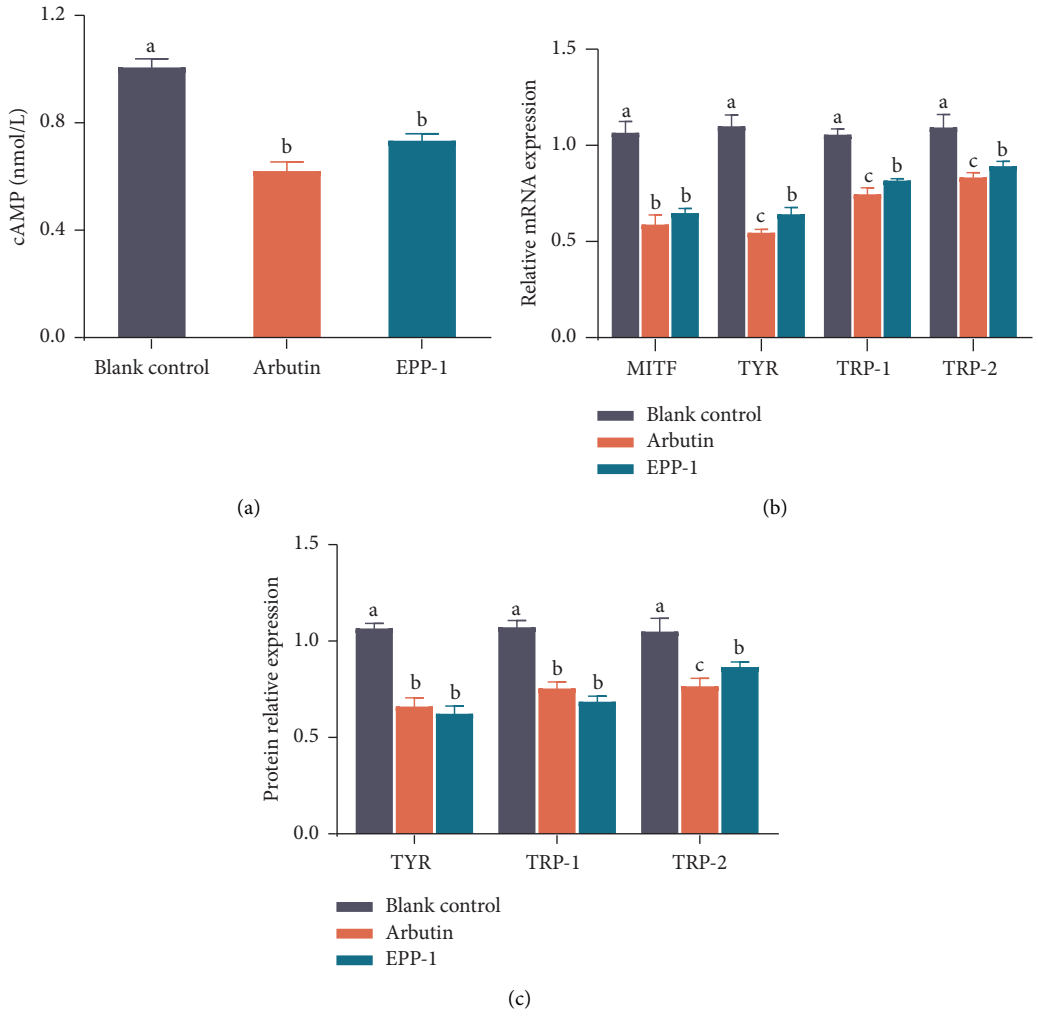

Figure 4: Effect of EPP-1 on the expression levels of melanin-related genes in B16-F10 melanoma cells: (a) the content of cAMP in B16-10 cells; (b) relative gene expression of MITF, TYR, TRP-1, and TRP-2; (c) relative protein expression of TYR, TRP-1, and TRP-2. Values with different letters under the same gene were significantly different between the columns ($P < 0.05$).

As shown in Figure 4(b), the mRNA expression levels of MITF, TYR, TRP-1, and TRP-2 were significantly decreased by stimulation with EPP-1, compared with that in the control group ($P < 0.05$). TYR, TRP-1, and TRP-2 mRNA expression were reduced by 35.83%, 18.36%, and 10.83%, respectively. Moreover, MITF mRNA expression was reduced by 35.16%. Therefore, it is hypothesized that EPP-1 can inhibit the expression of tyrosinase-related genes by downregulating the MITF gene.

Melanogenic enzymes such as TYR, TRP-1, and TRP-2 play an important role in melanin synthesis, and enzyme-linked immunosorbent assays were performed to determine the expression of these proteins. As shown in Figure 4(c), among the expression of tyrosinase family proteins, the protein expression levels of TYR, TRP-1, and TRP-2 were significantly decreased under the stimulation of EPP-1 ($P < 0.05$), with inhibition rates of 37.67%, 31.46%, and 13.46%, respectively.

4. Discussion

EBN, an Asian health food, is rich in nutritional components [8, 24] like high protein, carbohydrates, and sialic acid and is known as "Caviar of the East" [25]. In this study, the whitening activity of EBN digestion products was investigated by murine melanocytes cell model, tyrosinase inhibitory activity, melanin production, and the expression levels of melanin-related genes were analyzed.

Protein is considered a vital factor in nutrition and medicinal value of EBN [26]. Pepsin and pancreatin were used to simulate the human gastrointestinal digestion environment and to maximize the digestion of the large molecular proteins contained in EBN. Peptides with low molecular weight are more readily absorbed and utilized by the human body than complete proteins [12]. Protein hydrolysates and peptides from nature sources were reported with a skin-whitening effect [27]. Deng et al. found that the

molecular weight of peptides with whitening activity was mostly below 3 kDa, and their whitening activity was related to a specific structure in peptide chains [28]. Pongkai et al. discovered that the chicken feather meal peptides of <3 kDa exhibited strong tyrosinase inhibition activity [29]. Therefore, this study investigated the small molecular weight fraction activity during the simulated digestion products.

In the biosynthesis pathway of melanin, tyrosinase is the speed-limiting enzyme that catalyzes oxidation. Therefore, tyrosinase is a key target for screening melanin synthesis inhibitors [30]. Tyrosinase inhibition and sialic acid contents of digested EBN products gradually increased during the simulation of gastrointestinal digestion *in vitro* (Figures 1(a)–1(c)). EBN is rich in sialic acid. Sialic acid with a nine-carbon ketoacid-neuraminic acid backbone can exist in free form in organisms and is also frequently attached to the ends of glycoconjugates as an important component; it is a vital material basis for the structural and functional diversification of glycoconjugates [31, 32]. Sialic acid has been found to inhibit tyrosinase activity and reduce the ability of L-dopa to convert into dopachrome [33]. N-acetylneuraminic acid as the main form of sialic acid in EBN showed skin-whitening function [10].

Furthermore, the molecular weight distribution of digested EBN products during simulated gastrointestinal digestion was measured (Figure 1(d)); simulated gastrointestinal digestion accelerated the hydrolysis of macromolecular protein and increased the content of small molecular peptides. EBN proteins are absorbed and utilized in the human body, mainly in the form of oligopeptides after digestion [34]. The molecular weight of the digestion products of EBN was mainly distributed at <1 kDa after simulated digestion experiment *in vitro*. Meanwhile, the tyrosinase inhibition of different molecular weight fractions was measured, and the highest tyrosinase inhibition activity was <1 kDa fractions (Figure 2). This is consistent with the previous studies that have found that small peptides have the whitening activity. Also, it may be related to the structure of peptide chain. It has been reported that a peptide with a molecular weight of 526 Da isolated from marine microalgae could inhibit α-MSH-induced melanogenesis in B16-F10 melanoma cells [35]. In this study, we found that EPP-1 significantly decreased intracellular melanin content and tyrosinase activity by measuring melanin content and tyrosinase activity in B16-F10 cells ($P < 0.05$).

The safe concentrations of EPP-1 were determined by an MTT experiment in B16-F10 cells. As shown in Figure 3(a), when the content of EPP-1 was 0.5–4 mg/mL, the inhibitory effect of EPP-1 on cells was not significant ($P > 0.05$). As shown in Figures 3(b) and 3(c), intracellular tyrosinase inhibition and melanin content were significantly decreased after treatment with EPP-1. The cAMP is an intracellular second messenger, and its alteration could regulate the expression level of target proteins in the melanogenic signaling pathway and result in an inhibitory effect on intracellular melanogenesis [36]. As shown in Figure 4(a), compared to the blank control group, EPP-1 significantly reduced the intracellular cAMP content. In this study, we determined whether EPP-1 regulated melanin content and tyrosinase activity by regulating the expression of genes associated with melanin synthesis. The expression levels of MITF and cAMP were analyzed, which play an important role in regulating melanin synthesis [37], and the expression of tyrosinase family genes (TYR, TRP-1, and TRP-2) was measured. Our data indicated that EPP-1 could inhibit melanogenesis by suppressing the cAMP/MITF/TRP-1/TRP-2/tyrosinase pathway (Figures 4(b) and 4(c)). Based on the above experimental results, we found that the low molecular weight of EBN digestive juice had a better whitening activity. Therefore, the characterization of the composition and chemical structure of the whitening active ingredients in EPP-1 will be our further research goal.

5. Conclusion

EBN is widely consumed as a traditional health and medicinal food, rich in protein and sialic acid. Experiments have shown that the main component of EBN digestion product is <1 kDa (EPP-1) fraction, which has better whitening activity, and its tyrosinase inhibition rate reached 83.12% and peptides content was 92.44%. The results of the murine melanocytes cell model indicated that EPP-1 inhibited melanogenesis in a dose-dependent way, and the tyrosinase activity and melanogenesis were inhibited by downregulating the expression of cAMP signaling pathway and melanin-related factors (MITF, TYR, TRP-1, and TRP-2). Therefore, EPP-1 is expected to be used as a natural whitening agent in health products.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Haili Yi and Meizi Piao contributed equally to this manuscript.

Acknowledgments

This work was supported by the Natural Science Foundation of Shandong Province of China (ZR2021QC042) and the High-level Talent Research Fund of Qingdao Agricultural University (665/1120037).

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