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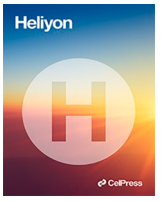

Research article

Employing fruit juices to hydrolyze edible bird's nest and enhance the antioxidant, anti-tyrosinase, and wound-healing activities of the hydrolysates

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ABSTRACT

Enzymatic hydrolysis of edible bird's nest (EBN) has attracted great interest in both scientific and commercial fields due to the enhancement of solubility and nutraceutical values. The present study attempted to investigate the hydrolysis of EBN with papaya (*Carica papaya* L.), pineapple (*Ananas comosus* (L.) Merr.), and cantaloupe (*Cucumis melo* L.) juices as well as two commercial enzymes papain and bromelain. Our analysis revealed that EBN hydrolysis with pineapple juice and bromelain produced a degree of hydrolysis (DH) value of approximately 27 % while it was about 25 % for the hydrolysis with cantaloupe juice and 22 % for the hydrolysis with papaya juice and papain after 4 h of treatment. When EBN was digested by fruit juices and enzymes, the protein solubility and free sialic acid content were increased and the highest values were achieved for EBN hydrolysis with pineapple juice and bromelain (estimately 11 mg/mL of soluble protein and 18 g/kg of free sialic acid). The $ABTS^{\bullet+}$-scavenging, $^{\bullet}OH$-scavenging, and anti-tyrosinase capacities were higher in the EBN hydrolysates by papaya juice (IC_{50} of 0.034, 0.108, and 0.419 mg/mL, respectively), pineapple juice (IC_{50} of 0.025, 0.045, and 0.190 mg/mL, respectively), and cantaloupe juice (IC_{50} of 0.031 mg/mL, 0.056, and 0.339 mg/mL, respectively) than in the hydrolysates by unhydrolyzed EBN (IC_{50} of 0.094, 0.366, and 1.611 mg/mL, respectively). An improvement in $ABTS^{\bullet+}$-scavenging, $^{\bullet}OH$-scavenging, and anti-tyrosinase abilities was also observed for the hydrolysates by papain (IC_{50} of 0.041, 0.129, and 0.417 mg/mL, respectively) and bromelain (IC_{50} of 0.025, 0.069, and 0.336 mg/mL, respectively) but in a lesser extent as compared to the hydrolysates by respective papaya and pineapple juices. Noticeably, the EBN hydrolysates by fruit juices remarkably enhanced the wound closure in human fibroblasts by about 1.4–1.8 times after 24 h of treatment whereas this property was insignificant in the hydrolysates by enzymes. As papaya, pineapple, and cantaloupe juices are easily obtainable and have pleasant flavors, our results provide a possible method to hydrolyze EBN and apply the resultant hydrolysates in functional food products.

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1. Introduction

Enzymatic hydrolysis of animal or plant proteins has recently gained immerse interest due to their superior advantages. The method not only offers a precise control of protein hydrolysis degree but also limits the loss and chemical modification of amino acids after the reaction. In addition, the low amount of utilized enzymes and the simplicity of deactivating procedures facilitate downstream steps of hydrolysate purification [1]. The resultant hydrolysates produced from enzymatic hydrolysis are attractive sources in providing nutritional and physiological peptides. Two peptides, Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP), for example, isolated from the hydrolysis of milk protein, were able to reduce hypertension and improve arterial function in rats [2]. The hydrolysis of soy protein isolate with Alcalase generated another peptide Phe-Asp-Pro-Ala-Leu (FDPAL, 561 Da) exhibiting antioxidant activity in HeLa cells as well as *Caenorhabditis elegans* [3]. Hence, the enzymatic approach of converting proteins from different sources into high-quality hydrolysates provides promising applications in food and health products [4].

Edible bird's nest (EBN), a salivary product of swiftlets *Aerodramus* and *Collocalia*, is a well-known tonic delicacy in Asian countries [5]. It contains a substantial amount of glycoprotein (more than 60 % of the EBN dry weight) [6]. Glycoprotein consisting of sialic acid significantly contributes to the pharmacological properties of EBN such as the antiviral, anti-tyrosinase, and wound-healing activities [7–9]. However, only 5 % of EBN protein is extracted by the traditional stewing/boiling method, preventing effective digestion of EBN protein in the human body [10]. In order to increase the solubility of EBN proteins, enzymatic approaches have been developed in recent years. In a study by Zulkifli et al., protein solubility was improved by more than 100-fold upon the application of Alcalase and papain after 3 h [11]. In addition, enzymatic treatment also produces EBN hydrolysates with enhanced nutraceutical properties compared to boiled EBN [10,12–14]. For example, Alcalase-digested EBN exhibited higher abilities to scavenge free radicals and inhibit angiotensin-I converting enzyme (ACE), an enzyme involved in the regulation of blood pressure, than undigested EBN [13]. EBN hydrolysate treated with simulated gastric fluid showed stronger inhibition of tyrosinase activity and melanin level in B16 cells, suggesting the potential of the hydrolysate in skin whitening [14]. Similarly, the addition of alkaline protease to EBN produced peptides with anti-photoaging capacity in zebrafish and wound-healing ability in MRC-5 cells [12]. These results indicate the advantage of the enzymatic hydrolysis over the conventional stewing/boiling method and the application potential of EBN hydrolysates in healthcare products.

Pure and crude proteolytic enzymes derived from animals, plants, fungi, and bacteria are often used to hydrolyze EBN protein [10–15]. As proteolytic enzymes can differ in cleavage sites, the combination of proteolytic enzymes can sometimes be applied to generate protein hydrolysates with more diverse peptides and ultimately more attractive properties than individual enzymes. For instance, hazelnut protein isolate treated with both Alcalase and Neutrase displayed a higher antioxidant capacity than that treated with individual Alcalase and Neutrase [16]. The utility of two enzymes (pepsin-papain, papain-trypsin and papain-pancreatin) has been reported for EBN hydrolysis. Unfortunately, these combinations increased the protein solubility less efficiently than single papain [17]. Other bioactivities of EBN hydrolysates by enzyme combinations have not been thoroughly evaluated. As fruit juices contain multiple native proteases, they can be promising sources to effectively improve the bioactivities of EBN hydrolysates. Additionally, new bioactive peptides generated under the cleavage of multiple native proteases can also be appealing candidates for further investigations.

Here, we investigated the effect of several fruit extracts on the hydrolysis of EBN. Besides, we assessed the antioxidant, anti-tyrosinase, and wound-healing abilities of obtained EBN hydrolysates as these properties are attractive in health and beauty products. Particularly, antioxidant, anti-tyrosinase, and wound-healing peptides derived from the protein hydrolysates have captured great interest due to their potential against oxidative stress-related diseases, melanin-related skin disorders, and skin injuries [18–20]. Popular and flavory fruits in Asian countries where EBN is widely consumed such as pineapple (*Ananas comosus (L.) Merr.*), papaya (*Carica papaya* L.), and cantaloupe (*Cucumis melo* L.) were selected for the experiments [21–24]. Pineapple juice contains a mixture of closely-related proteolytic enzymes in which four cysteine proteases (fruit bromelain, stem bromelain, ananain, and comasain) have been identified [22]. Papaya fruit extract is also comprised of four major cysteine proteases, including papain, chymopapain, glycyl endopeptidase, and caricain while serine proteases such as cucumisin and protease D are present in the sarcocarp of melon fruit [21,23,24]. Recently, the EBN hydrolysate prepared from papaya juice digestion has been shown to inhibit the activity of α-glucosidase, a hyperglycemia-associated enzyme. Meanwhile, this effect has not been observed in other two EBN digests produced by Alcalase and papain enzymes [11]. Pineapple and cantaloupe juices have not been investigated for the hydrolysis of EBN; however, they were applied for meat, soybean, and kilka fish proteins [25,26]. Importantly, it was reported that the antioxidant property was better in a hydrolysate of kilka fish protein by melon extract than by Alcalase [25]. The abundance of proteases as well as the historical use of these fruits suggest their potential in digesting EBN proteins and producing hydrolysates with valuable bioactivities.

2. Material and methods

2.1. Preparation of EBN hydrolysates by fruit juices, papain, and bromelain

Farmed EBN was kindly provided by Phuoc Tin Development Trading Service Company, Limited, Vietnam. The preparation of EBN slurry and fruit juices was performed similarly to previous reports with some modifications [11,27]. Briefly, EBN (2 g) was boiled with distilled water for 3 h to achieve a final volume of 100 mL. Fresh, ripe yellow-fleshed papaya (*Carica papaya* L.), pineapple (*Ananas cosmosus* (L.) Merr.), and cantaloupe (*Cucumis melo* L.) (100 g) harvested in Southern Vietnam were peeled and crushed in a mortar. Subsequently, the juices were centrifuged at 6000 rpm for 10 min at 10 °C to obtain start supernatants (about 50–60 mL), designated hereafter as PJ, AJ, and CJ for papaya, pineapple, and cantaloupe juices, respectively.

EBN hydrolysates by fruit juices were produced by adding a volume of 2.5 mL of PJ, AJ, and CJ to 100 mL of the EBN slurry (juice to substrate ratio of 2.5:2 v/w). The conditions of reactions were set to a temperature of 60 °C for 4 h, and a pH of approximately 6.5 for PJ, 5.5 for AJ, and 8.5 for CJ during the hydrolysis. Heat treatment at 90 °C for 20 min and centrifugation at 8000 rpm for 10 min at 10 °C were then applied to inactivate proteases in the fruit juices and stop the hydrolysis reaction. The resultant EBN hydrolysates by papaya, pineapple, and cantaloupe juices, hereafter designated as HPJ, HAJ, and HCJ 4 h, respectively, were subjected to subsequent experiments. Mixtures of EBN with papaya, pineapple, and cantaloupe juices were referred to as HPJ, HAJ, and HCJ 0 h, respectively. Three independent hydrolysates by fruit juices were prepared for subsequent experiments.

Papain and bromelain (Biogreen BPC., JSC, Vietnam) at the enzyme-to-substrate ratio of 1.25:2 and 0.625:2 w/w, respectively, were used to hydrolyze EBN at a temperature of 60 °C, time of 4 h, and a pH of about 6.5 for papain and 7.0 for bromelain [11,28]. Similar steps were carried out as aforementioned for the EBN hydrolysis with fruit juices to achieve EBN hydrolysates by papain (HPP 4 h) and bromelain (HBM 4 h). Mixtures of EBN with papain and bromelain were referred to as HPP and HBM 0 h, respectively. Three independent hydrolysates by papain and bromelain were employed for further assays.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples subjected to SDS-PAGE (EBN, HPJ, HAJ, HCJ, HPP, and HBM 4 h) were mixed with protein loading buffer. After denaturing proteins by boiling for 5 min, the samples were loaded on the wells of 15 % SDS-PAGE gel. Gel staining was performed with Coomassie blue to detect protein bands in the samples [29].

2.3. Determination of total protease activity of fruit juices

Total protease activity of PJ, AJ, and CJ on casein was quantified based on the amount of free tyrosines released from the substrate [30]. Blue chromophores produced by the reaction of free tyrosines with Folin-Ciocalteau phenol reagent were determined by the absorbance in a UV spectrophotometer (Genway, USA). Tyrosine (Sigma-Aldrich, Germany) was used to build a standard curve. The total protease activity of fruit juices (U/mL) as the amount in micromoles of tyrosine standard equivalents released from casein per min was calculated by the formula:

$$\text{Protease activity (U/mL)} = [(\mu\text{mol tyrosine equivalents released}) \times (A)]/[(B) \times (C) \times (D)]$$

Where.

A: total volume of assay (mL)
B: time of assay (min)
C: volume of enzyme (mL)
D: volume in colorimetric determination (mL)

2.4. Determination of degree of hydrolysis

The O-phthalaldehyde (OPA) method was used to quantify the degree of hydrolysis (DH) of test samples as previously described [31]. Briefly, 200 μL of freshly prepared OPA was added into 25 μL of the samples (HPJ, HAJ, HCJ, HPP, and HBM 4 h) and serine standard (0.9516 meqv/L) in a 96-well Optiplate (PerkinElmer, USA). Subsequently, an incubation for 5 min was performed in the dark. Absorbance was read at 340 nm in a spectrophotometer (Genway, USA).

The DH of samples was calculated by the formula:

$$DH\ (\%) = (H/H_{tot}) \times 2\%$$

Where,

$$H\ (\text{meqv/g protein}) = (\text{SerineNH}_2 - \beta)/\alpha$$

With,

$$\text{SerineNH}_2\ (\text{meqv/g protein}) = [(A_{sample} - A_{blank})/(A_{standard} - A_{blank})] \times 0.9516 \times V \times [(2/(X \times P)]$$

A_{sample}: Absorbance of the sample
$A_{standard}$: Absorbance of serine standard
V: Volume of the sample
X: Amount of the sample
P: Percentage of protein in the sample (60 % for EBN)
0.9516 (meqv/L): Equivalent weight of serine standard
H_{tot} = 7.8 meqv/g protein, α = 1, and β = 0.34 [31]

2.5. Determination of protein solubility in EBN hydrolysates

The content of soluble protein in the test samples (unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ at 4 h of hydrolysis) was determined as previously described [32]. Bovine serum albumin (BSA, Sigma-Aldrich, Germany) was used to build a standard curve. A volume of 100 μL of Bradford reagent was added into 100 μL of the test sample and BSA in a 96-well plate. After incubating for 10 min at room temperature, absorbance was read at 595 nm in a microplate reader (Biotek ELX800, USA). Three independent experiments were carried out for the assay.

2.6. Determination of free sialic acid in EBN hydrolysates

Test samples (unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ at 0 and 4 h of hydrolysis) were sent out for analyzing free sialic content at Center for Analytical Services and Experimentation (CASE, Vietnam), a certified analytical center. Briefly, 2 μL of each sample was directly injected into a C18 column (Waters, USA) of liquid chromatography with tandem mass spectrometry (Agilent 6410 Triple Quad MS/MS system, Agilent, USA). The mobile phase consisted of 0.1 % formic acid and 0.1 % acetonitrile. Two independent experiments were carried out for the assay.

2.7. In vitro antioxidant activity

The assay to determine the antioxidant activity using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma-Aldrich, Germany) was conducted as previously described [33]. Test samples (unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ from the hydrolysis at 0 and 4 h) were diluted to obtain various concentrations of initial EBN (0.00625, 0.0125, 0.025, 0.05, and 0.1 mg/mL). A volume of 900 μL $ABTS^{\bullet+}$ solution was added to 100 μL of diluted test samples and further incubated for 15 min. The $ABTS^{\bullet+}$-scavenging ability expressed by the absorbance at 734 nm was calculated as follows:

$$ABTS^{\bullet+}\text{-scavenging capacity (\%)} = 100 - 100 \times (A_2 - A_3)/A_1$$

Where.

A_1: Absorbance of $ABTS^{\bullet+}$ only (negative control)
A_2: Absorbance of the sample after reacting with $ABTS^{\bullet+}$
A_3: Absorbance of the sample without $ABTS^{\bullet+}$ (blank)

Another *in vitro* antioxidant assay based on Fenton reaction was used to evaluate the hydroxyl radical ($^{\bullet}OH$)-scavenging capacity of EBN hydrolysates [34]. Initially, 50 μL of 9 mM iron (II) sulfate ($FeSO_4$) in 10 mM of ethylenediaminetetraacetic acid (EDTA) was mixed with 100 μL of 8 mM hydrogen peroxide (H_2O_2) in a 96-well plate. This step was followed by the addition of 100 μL of the diluted unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ from the hydrolysis at 0 and 4 h containing 0.063, 0.125, 0.188, 0.25 mg/mL of EBN. After that, the plate was incubated with 20 μL of 9 mM of salicylic acid and read at 510 nm in a microplate spectrophotometer (VICTOR Nivo 3F, PerkinElmer, USA). The $^{\bullet}OH$-scavenging capacity was calculated by the formula:

$$^{\bullet}OH\text{-scavenging capacity (\%)} = 100 - 100 \times (A_2\text{-}A_3)/A_1$$

Where.

A_1: Absorbance of negative control (Milli-Q water)
A_2: Absorbance of the sample after reacting with salicylic acid
A_3: Absorbance of the sample without salicylic acid

The half-maximal inhibitory concentration (IC_{50}) of sample (the concentration of sample which inhibits 50 % of the $ABTS^{\bullet+}$- or $^{\bullet}OH$ -radicals) was calculated by GraphPad Prism software (version 5.0, Insightful Science LLC, USA).

2.8. Anti-tyrosinase assay

The assay was based on the formation of L-dopachrome by the action of mushroom tyrosinase enzyme on its substrate, L-tyrosine (Sigma-Aldrich, Germany) [35]. An aliquot of 10 μL of tyrosinase (250 U/mL, Sigma-Aldrich, Germany) was supplemented to the wells of a 96-well plate containing 60 μL of diluted unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ from the hydrolysis at 0 and 4 h at the initial EBN concentrations of 0.0325, 0.075, 0.15, 0.3, and 0.6 mg/mL. This step was followed by the incubation of the plate at room temperature for 20 min and the addition of 140 μL of L-tyrosine (0.3 mg/mL) in phosphate buffer (pH 6.8). The absorbance of L-dopachrome at 480 nm was monitored in a microplate reader (VICTOR Nivo Filter, PerkinElmer, USA). The anti-tyrosinase ability of the samples was calculated as follows:

$$\text{Tyrosinase inhibitory capacity (\%)} = 100 \times [(B_1\text{–}B_3)\text{-}(B_2\text{–}B_4)]/(B_1\text{–}B_3)$$

Where.

B_1: Absorbance of L-tyrosine (negative control)
B_2: Absorbance of the sample after reacting with tyrosinase
B_3 and B_4: Absorbance of the blank for the negative control and sample, respectively.
The IC_{50} value of sample for the anti-tyrosinase capacity was calculated by GraphPad Prism software (version 5.0, Insightful Science LLC, USA).

2.9. In vitro scratch assay

The assay was performed on the human fibroblast model as previously described [36]. Briefly, fibroblast cells were cultured in DMEM high glucose medium, 10 % fetal bovine serum (FBS) (HyClone, Cytiva, USA) for 24 h at 37 °C with 5 % CO_2. Scratched wounds were generated by a 10 μl pipette tip followed by the addition of new DMEM high glucose medium and 5 % (v/w) of test samples (unhydrolyzed EBN, HPJ, HPP, HAJ, HBM, and HCJ from the hydrolysis at 0 and 4 h). Distilled water served as a negative control. The wound images were captured at 0, 12, and 24 h and the wound areas were measured in Image J software. The wound-healing activity of the samples was quantified by the formula:

$$\text{Wound closure (\%)} = 100 \times (C_1 - C_2)/C_1$$

Where.

C_1: the wound area of the sample at 0 h
C_2: the wound area of the sample after 12 and 24 h

2.10. Statistical analysis

Analyses were conducted in two or three independent experiments. One-way analysis of variance (ANOVA) with Tukey's posttest was used to calculate statistical differences among samples, except for the analysis of free sialic acid content in which Student's t-test was applied. p values were considered significant at $p \leq 0.05$.

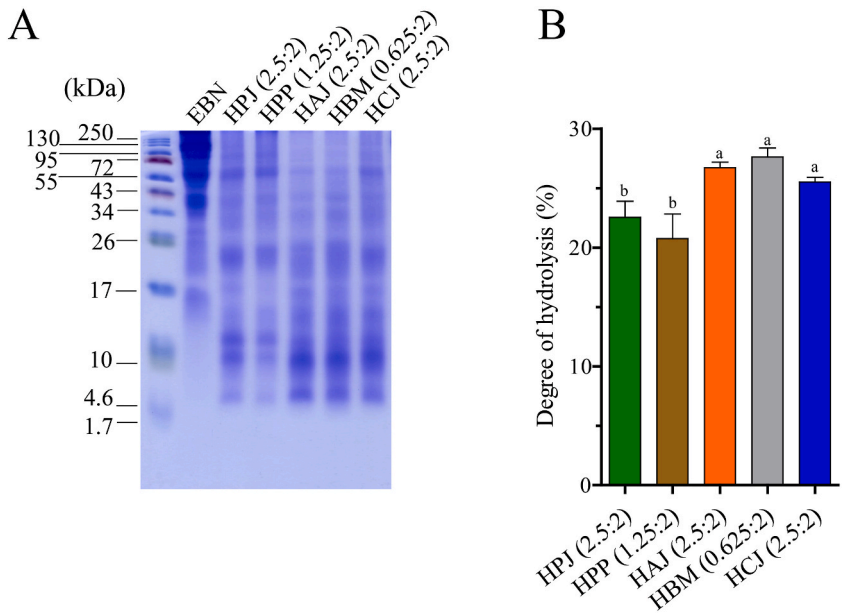

Fig. 1. Hydrolysis of EBN by fruit juices, papain, and bromelain. (A) SDS-PAGE separation of proteins and peptides in unhydrolyzed EBN and EBN hydrolysates by fruit juices, papain, and bromelain. (B) Degree of hydrolysis of EBN by the treatment with fruit juices, papain, and bromelain. Each data bar is shown as mean ± SD (n = 3). Different letters indicated statistical differences among samples with $p \leq 0.05$ in one-way ANOVA. HPJ, HAJ, and HCJ: EBN hydrolysates by papaya, pineapple, and cantaloupe juices (juice to substrate ratio of 2.5:2 v/w), respectively; HPP: EBN hydrolysate by papain (enzyme to substrate ratio of 1.25:2 w/w); HBM: EBN hydrolysate by bromelain (enzyme to substrate ratio of 0.625:2 w/w). The hydrolysates were obtained at 4 h of hydrolysis. The uncropped image of the SDS-PAGE gel is provided in Supplementary Fig. 1.

3. Results and discussion

3.1. The hydrolysis of EBN by fruit juices, papain, and bromelain

As seen on the SDS-PAGE gel (Fig. 1), EBN was detected as a smear, distributed from more than 250 kDa down to approximately 10 kDa, suggesting a diversity of proteins present in EBN. In addition, thick bands showed up from 34 to 250 kDa in which bands in the molecular weight ranges of 95–130 kDa were described as sialic acid-binding glycoprotein [37]. After the treatment with fruit juices (juice to substrate ratio of 2.5:2 v/w), papain (enzyme to substrate ratio of 1.25:2 w/w), and bromelain (enzyme to substrate ratio of 0.625:2 w/w), large and complex proteins were hydrolyzed into smaller molecules as indicated by the faint high-molecular-weight bands at more than 34 kDa and the appearance of low-molecular-weight bands, especially bands in 17–26 kDa and 4.6–10 kDa ranges. Papain and bromelain, which have been previously reported for their hydrolysis of EBN, were able to break down EBN into a relatively similar pattern as papaya and pineapple juices, respectively (Fig. 1A and B) [11,13].

The decrease of high-molecular-weight proteins and the increase of low-molecular-weight proteins in HAJ, HBM, and HCJ were higher than those in HPJ and HPP, implying a stronger hydrolysis of EBN by pineapple, bromelain, and cantaloupe juices than by papaya juice and papain (Fig. 1A). Consistently, DH values of EBN reached approximately 27 % for pineapple juice and bromelain and 25 % for cantaloupe juice while a lower DH value (about 22 %) was obtained by the application of papaya juice and papain (Fig. 1B). The difference in the hydrolysis performance between fruit juices might be attributed to their protease activity. Our analysis revealed that the total protease activity of pineapple and cantaloupe juices was indeed higher than that of papaya juice (14.06 $\pm$ 0.12, 3.20 $\pm$ 0.09, 2.62 $\pm$ 0.07 U/mL for pineapple, cantaloupe, and papaya juices, respectively). In a study by Zulkifli et al., a DH of 16 % was obtained by the hydrolysis of EBN with papaya juice (juice to substrate ratio of 60:2 w/w) after 3 h [11]. Our study applied a lower papaya juice to substrate ratio to achieve a DH of 22 % after 4 h of hydrolysis. This might be explained by the difference in the proteolytic activities of papaya sources.

Although most of EBN was hydrolyzed after 4 h of treatment with fruit juices, papain, and bromelain, a small amount of protein remained undegraded (Fig. 1A). The incomplete hydrolysis of EBN, which was also previously observed for pepsin even with the incubation time of 48 h, might be resulted from the low ratio of proteases or the conditions of hydrolysis [14]. However, macropeptides (5–10 kDa) could be achieved in all EBN hydrolysates despite the undetectable amount of peptides less than 4 kDa as in the former EBN hydrolysates by pepsin or alkaline protease [12,14]. These macropeptides can serve as bioactive peptides and contribute to the enhanced functional properties of our EBN hydrolysates.

3.2. Protein solubility in EBN hydrolysates by fruit juices, papain, and bromelain

Protein was soluble at the concentration of about 1 mg/mL in EBN by our boiling method. After digestion with fruit juices and enzymes, the solubility of protein was elevated in all samples as compared to EBN. The highest values were achieved for HAJ and HBM (11 mg/mL), followed by HCJ (10 mg/mL), HPJ (9 mg/mL), and HPP (9 mg/mL) (Fig. 2). These results indicated the improvement of

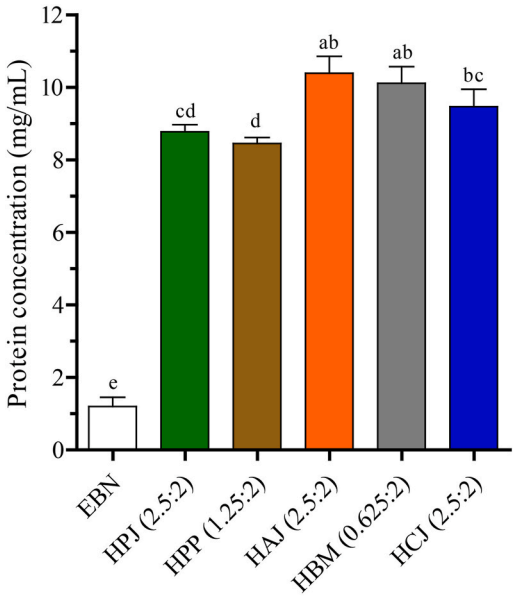

Fig. 2. The protein solubility of the EBN hydrolysates by fruit juices, papain, and bromelain. Each data bar is shown as mean $\pm$ SD (n = 3). Different letters indicated statistical differences among samples with $p \leq 0.05$ in one-way ANOVA. HPJ, HAJ, and HCJ: EBN hydrolysates by papaya, pineapple, and cantaloupe juices (juice to substrate ratio of 2.5:2 v/w), respectively; HPP: EBN hydrolysate by papain (enzyme to substrate ratio of 1.25:2 w/w); HBM: EBN hydrolysate by bromelain (enzyme to substrate ratio of 0.625:2 w/w). The hydrolysates were obtained at 4 h of hydrolysis.

protein solubility in EBN by the hydrolysis with our three experimental fruit juices and two commercial enzymes, papain and bromelain.

An elevation in the content of soluble protein by the digestion of EBN with papain and papaya juice was also previously reported [11]. It is possible that enzymatic hydrolysis might influence the distribution of hydrophobic and hydrophilic amino acids on the surface, the protein-water interaction, and eventually increase the protein solubility. As soluble proteins and peptides are well-digested and quickly absorbed by the human body, EBN hydrolysates were much favored by athletes or digestive-impaired persons [37]. Therefore, our hydrolysates produced by fruit juices hold a promise in application due to the convenience and availability of fruits as compared to commercial enzymes.

3.3. Antioxidant activity of EBN hydrolysates by fruit juices, papain, and bromelain

In this study, the antioxidant capacities of EBN hydrolysates by fruit juices, papain, and bromelain assays were assessed by the $ABTS^{\bullet+}$- and $^{\bullet}OH$-scavenging assays. Our data indicated that hydrolysis with papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain improved the antioxidant property of EBN. For example, at the EBN concentration of 0.05 mg/mL, HPJ, HAJ, HCJ, and HPP 4 h were able to scavenge approximately 60–70 % of $ABTS^{\bullet+}$ radicals while the scavenging values were only around 30 % for unhydrolyzed EBN and 40 % for HPJ, HAJ, HCJ, and HPP 0 h. In the case of the treatment with bromelain, 60 % of $ABTS^{\bullet+}$ radicals were captured in HBM 0 h, lower than in HBM 4 h (80 %) (Fig. 3A). In the $^{\bullet}OH$-scavenging assay, HPJ 4 h and HPP 4 h inhibited the $^{\bullet}OH$ radicals (about 70–75 %) more efficiently than unhydrolyzed EBN (40 %), HPJ, and HPP 0 h (about 55 %) at the EBN concentration of 0.25 mg/mL. At this concentration of EBN, the $^{\bullet}OH$-scavenging abilities of HAJ, HBM, and HCJ 4 h (90 %, 85 %, and 95 %, respectively) were also higher than those of HAJ, HBM, and HCJ 0 h (about 70 %) (Fig. 3B). Consistently, the IC_{50} values for $ABTS^{\bullet+}$- and $^{\bullet}OH$-scavenging capacities of HPJ 4 h (0.034 and 0.108 mg/mL, respectively), HPP 4 h (0.041 and 0.129 mg/mL, respectively), HAJ 4 h (0.025 and 0.045 mg/mL, respectively), HBM 4 h (0.025 and 0.069 mg/mL, respectively), and HCJ (0.031 and 0.056 mg/mL, respectively) were lower than those of EBN (0.094 and 0.366 mg/mL, respectively), respective HPJ 0 h (0.068 and 0.189 mg/mL, respectively), HPP 0 h (0.060 and 0. 0.181 mg/mL, respectively), HAJ 0 h (0.081 and 0.120 mg/mL, respectively), HBM 0 h (0.043 and 0.113 mg/mL, respectively), and HCJ 0 h (0.069 and 0.136 mg/mL, respectively) (Table 1).

The enhanced antioxidant activity of EBN hydrolysates by fruit juices, papain, and bromelain might be resulted from the antioxidant peptides generated during the hydrolysis process. Antioxidant peptides have been known to exert their antioxidative function mainly via inactivation of reactive oxygen species, scavenging of free radicals, chelation of prooxidative transition metals, and reduction of hydroperoxides [38]. Our assay demonstrated that trapping of free radicals is one potential mechanism employed by antioxidant peptides present in the EBN hydrolysates. This mechanism was also reported in other studies using alkaline protease, Alcalase, and papain for EBN hydrolysis [11,12,39,40].

At an equivalent DH, EBN hydrolysis with papaya and pineapple juices showed better $ABTS^{\bullet+}$- and $^{\bullet}OH$-scavenging capacities than that with papain and bromelain, respectively. Although a higher DH was obtained by the treatment with cantaloupe juice than with papaya juice, the scavenging capacities were not much different between the two EBN hydrolysates. The distinct behaviors of EBN hydrolysates toward antioxidancy could be originated from the characteristics of obtained bioactive peptides such as their molecular weight (length), amino acid composition, sequences, and hydrophobicities [41]. Previously, Bai et al. employed alkaline protease to produce an EBN hydrolysate with strong $DPPH^{\bullet}$ and $ABTS^{\bullet+}$-scavenging capacities (IC_{50} of 1.08 and 0.46 mg/mL, respectively). It was speculated in their study that the high contents of hydrophobic (His, Trp, Phe, Pro, Gly, Lys, Ile, and Val), aromatic (Trp, Tyr, and Pro), and acidic (Asp and Glu) amino acids might contribute to the antioxidant potency of the EBN hydrolysate [[12]]. Such hydrophobic and aromatic amino acids also appeared in the two antioxidant pentapeptides Pro-Phe-His-Pro-Tyr and Leu-Leu-Gly-Asp-Pro isolated

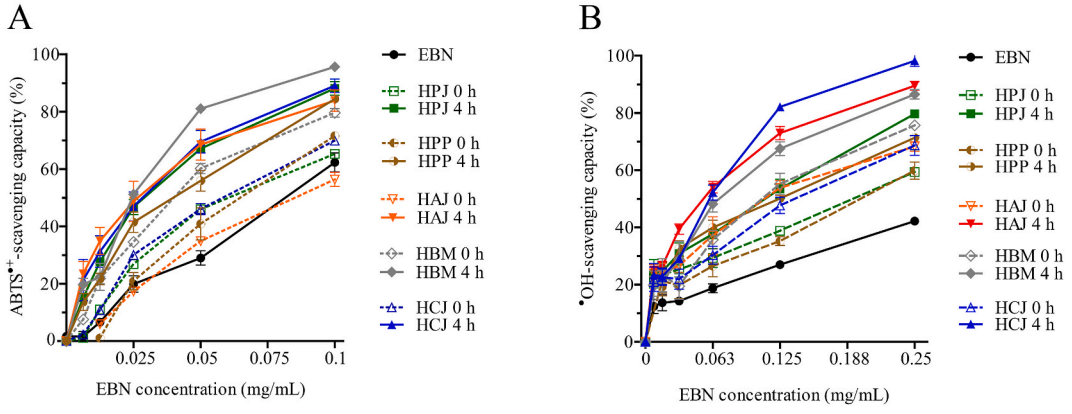

Fig. 3. The antioxidant activity of the EBN hydrolysates by fruit juices, papain, and bromelain. (A) The $ABTS^{\bullet+}$-scavenging capacity. (B) The $^{\bullet}OH$-scavenging capacity. Each data point is shown as mean ± SD (n = 3). HPJ 0 h, HAJ 0 h, HCJ 0 h, HPP 0 h, and HBM 0 h: mixtures of EBN with papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain, respectively. HPJ 4 h, HAJ 4 h, HCJ 4 h, HPP 4 h, and HBM 4 h: EBN hydrolysates by papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain at 4 h of hydrolysis, respectively.

Table 1
IC_{50} values (mg/mL) of EBN hydrolysates by fruit juices, papain, and bromelain for the $ABTS^{\bullet+}$-scavenging, $^{\bullet}OH$-scavenging, and tyrosinase inhibitory capacities.

Samples	IC_{50} (mg/mL)		
	$ABTS^{\bullet+}$-scavenging capacity (mg/mL)	$^{\bullet}OH$-scavenging capacity (mg/mL)	Tyrosinase inhibitory capacity (mg/mL)
EBN	0.094 ± 0.002^{a}	0.366 ± 0.034^{a}	1.611 ± 0.023^{a}
HPJ 0 h	0.068 ± 0.005^{b}	0.189 ± 0.020^{b}	0.860 ± 0.007^{b}
HPJ 4 h	$0.034 \pm 0.002^{d,f}$	0.108 ± 0.010^{c}	0.419 ± 0.067^{e}
HPP 0 h	0.060 ± 0.006^{e}	0.181 ± 0.022^{b}	0.607 ± 0.09^{d}
HPP 4 h	0.041 ± 0.002^{h}	0.129 ± 0.007^{c}	0.417 ± 0.011^{h}
HAJ 0 h	$0.081 \pm 0.005^{a,c}$	0.120 ± 0.013^{cd}	$0.622 \pm 0.010^{c,d}$
HAJ 4 h	$0.025 \pm 0.004^{f,g}$	0.045 ± 0.008^{e}	0.190 ± 0.027^{g}
HBM 0 h	$0.043 \pm 0.003^{d,h}$	0.113 ± 0.017^{cd}	$0.649 \pm 0.011^{c,d}$
HBM 4 h	$0.025 \pm 0.001^{f,g}$	0.069 ± 0.009^{e}	0.336 ± 0.010^{f}
HCJ 0 h	$0.069 \pm 0.005^{b,c,e}$	0.136 ± 0.005^{c}	0.647 ± 0.008^{c}
HCJ 4 h	0.031 ± 0.004^{f}	0.056 ± 0.004^{e}	0.339 ± 0.010^{f}

Each data value is presented as mean ± SD (n = 3).
HPJ 0 h, HAJ 0 h, HCJ 0 h, HPP 0 h, and HBM 0 h: mixtures of EBN with papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain, respectively.
HPJ 4 h, HAJ 4 h, HCJ 4 h, HPP 4 h, and HBM 4 h: EBN hydrolysates by papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain at 4 h of hydrolysis, respectively.

from an EBN pepsin-trypsin hydrolysate in a study by Ghassem et al. [[42]]. It would be interesting to identify bioactive peptides in the EBN hydrolysates by fruit juices and determine the participation of structural features in the antioxidative capacity in further studies.

It should be noted that HPJ, HCJ, HPP, and HBM 0 h exhibited stronger scavenging effects than EBN, suggesting the antioxidant activity of papaya juice, cantaloupe juice, papain, and bromelain. This property could be explained by the presence of polyphenol/flavonoids in the papaya, carotenoids/vitamin C in the cantaloupe, and the protein nature of papain and bromelain [43–46].

3.4. Anti-tyrosinase activity of EBN hydrolysates by fruit juices, papain, and bromelain

Our analysis showed that the hydrolysis with fruit juices, papain, and bromelain promoted the tyrosinase inhibitory effect of EBN. As shown in Fig. 4, 55–70 % of enzyme activity were inhibited in HPJ, HPP, HAJ, HBM, and HCJ 4 h while the inhibitory ability was only 20 % in EBN and around 30–40 % in HPJ, HPP, HAJ, HBM, and HCJ 0 h at the EBN concentration of 0.6 mg/mL. Accordingly, the IC_{50} values of HPJ (0.419 mg/mL), HPP (0.417 mg/mL), HAJ (0.190 mg/mL), HBM (0.336 mg/mL), and HCJ (0.339 mg/mL) at 4 h of hydrolysis were lower than those of EBN (1.611 mg/mL) and respective HPJ (0.860 mg/mL), HPP (0.607 mg/mL), HAJ (0.622 mg/mL), HBM 0.649 (mg/mL), and HCJ (0.647 mg/mL) at 0 h of hydrolysis (Table 1).

Fan et al. found that free sialic acid is a major active component responsible for the anti-tyrosinase activity of the EBN digested by simulated gastric and small intestinal enzymes. Free-form sialic acid accounts for about 63 % of the total anti-tyrosinase capacity, higher than protein and glycan-form sialic acid (approximately 27 % and 9 %, respectively) [7]. In addition to sialic acid, peptides in the protein hydrolysates could also participate in the inhibition of tyrosinase activity [47]. In our study, the improvement of tyrosinase inhibition in the EBN hydrolysates by fruit juices, papain, and bromelain was probably due to increased contents of free sialic acid

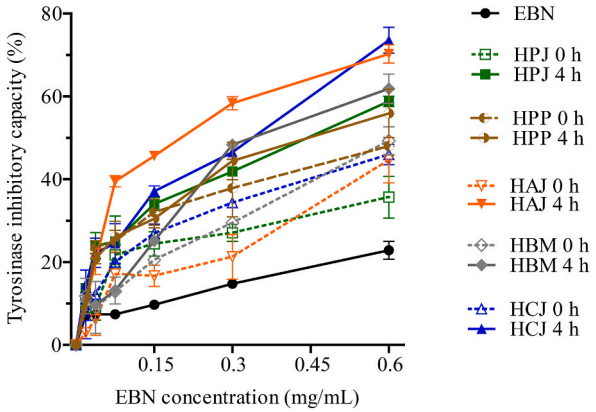

Fig. 4. The tyrosinase inhibitory capacity of the EBN hydrolysates by fruit juices, papain, and bromelain. Each data point is shown as mean ± SD (n = 3). HPJ 0 h, HAJ 0 h, HCJ 0 h, HPP 0 h, and HBM 0 h: mixtures of EBN with papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain, respectively. HPJ 4 h, HAJ 4 h, HCJ 4 h, HPP 4 h, and HBM 4 h: EBN hydrolysates by papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain at 4 h of hydrolysis, respectively.

generated during the digestion process or the presence of bioactive peptides with tyrosinase inhibitory effect. The significance of sialic acid and bioactive peptides toward the anti-tyrosinase capacity of the EBN hydrolysates by fruit juices, papain, and bromelain needs further investigation.

In our assay, better anti-tyrosinase capacities were obtained by the digestion with papaya and pineapple juices than respective papain and bromelain (Fig. 4 and Table 1). On the other hand, the potency to inhibit tyrosinase activity of the cantaloupe juice-treated hydrolysate is similar to that of the bromelain-treated one despite the fact that cantaloupe juice generated a lower DH value than bromelain. The differences in the anti-tyrosinase activity of EBN hydrolysates might be caused by the levels of free sialic acid or the properties of bioactive peptides (hydrophobicity, amino acid type, and the arrangement of terminal amino acid residues) in the hydrolysates [48].

It should be worth noting that the inhibitory effect on tyrosinase activity of HPJ, HAJ, and HCJ 0 h was stronger than that of EBN, indicating the self-anti-tyrosinase activity of papaya, pineapple, and cantaloupe juices (Fig. 4 and Table 1). Our observation is in agreement with reports by Shin et al., Le et al., and Boonpisuttinant et al. [49–51]. Although no literature for the tyrosinase inhibition of papain and bromelain has been found, our data clearly showed this property in both enzymes.

Tyrosinase is an important rate-limiting enzyme involved in melanogenesis and browning. Tyrosinase inhibitors preventing skin hyperpigmentation and fruit browning have attracted great interest due to their potential use in food and cosmetic industries [52]. Our study revealed the anti-tyrosinase activity of EBN hydrolysates by papaya, pineapple, and cantaloupe juices, making them promising tyrosinase inhibitors for further studies.

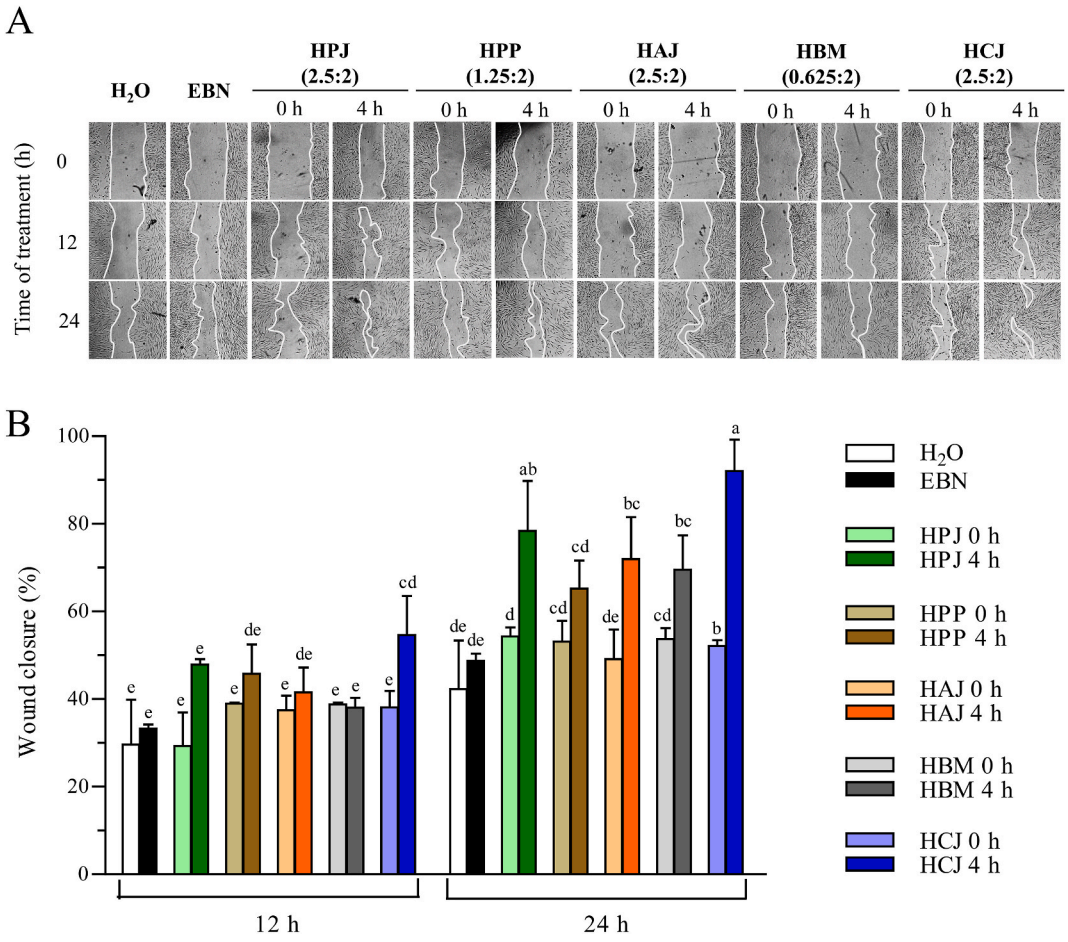

Fig. 5. The wound-healing activity of the EBN hydrolysates by fruit juices, papain, and bromelain. (A) Images of wound closure in human fibroblasts treated with H_2O, unhydrolyzed EBN, EBN hydrolysates by fruit juices, papain, and bromelain at 0, 12, and 24 h. (B) Quantification of wound closure (%) at 12 and 24 h. Each data bar is presented as mean ± SD (n = 3). Different letters indicated statistical differences among samples with $p \leq 0.05$ in one-way ANOVA. HPJ 0 h, HAJ 0 h, HCJ 0 h, HPP 0 h, and HBM 0 h: mixtures of EBN with papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain, respectively. HPJ 4 h, HAJ 4 h, HCJ 4 h, HPP 4 h, and HBM 4 h: EBN hydrolysates by papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain at 4 h of hydrolysis, respectively.

3.5. Wound-healing activity of EBN hydrolysates by fruit juices, papain, and bromelain

The *in vitro* scratch assay on human fibroblasts showed that the self-wound-healing process occurred in the H_2O-treated sample as the wound closed approximately 30 % at 12 h and 40 % at 24 h. Percentages of wound closure at 12 and 24 h in the samples treated with EBN, HPJ, HAJ, HCJ, HPP, and HBM 0 h were similar to those in the H_2O-treated sample, implying that unhydrolyzed EBN and mixtures of EBN with fruit juices and enzymes did not influence the rate of wound healing. On the contrary, the wound-healing activity of EBN hydrolysates by papaya, pineapple, and cantaloupe juices (HPJ, HAJ, and HCJ 4 h) was remarkably strong, as indicated by the percentages of wound closure up to 70–90 % at 24 h of treatment. Notably, the wound recovery was not significantly accelerated in HPP and HBM 4 h, implying that hydrolysis with papain and bromelain did not improve the wound-healing ability of EBN (Fig. 5A and B).

Bioactive peptides in the protein hydrolysates might be involved in the wound recovery as they can act on inflammation, epithelialization, tissue granulation and remodeling phases of the wound-healing process [18]. For example, collagen peptides prepared from the hydrolysis of jellyfish *Rhopilema esculentum* with alkaline protease/papain promoted scratch closure in human umbilical vein endothelial (HUVEC) cells and increased re-epithelialization, tissue regeneration, and collagen deposition by regulating the expression levels of β-fibroblast growth factor (β-FGF) and the transforming growth factor-β_1 (TGF-β_1) in mice model [53]. Since the wound-healing property is specific to the EBN hydrolysates by papaya, pineapple, and cantaloupe juices, it would be worthwhile to investigate the composition of bioactive peptides in these hydrolysates and their role in the wound healing in further *in vivo* studies.

3.6. Free sialic acid content of EBN hydrolysates by fruit juices, papain, and bromelain

As free sialic acid was proven to be a potent compound involved in the anti-tyrosinase activity of EBN, its content was determined in the EBN hydrolysates by fruit juices and enzymes [7]. Unhydrolyzed EBN and mixtures of EBN with fruit juices and enzymes contained approximately 4 g/kg (0.4 %) of free sialic acid. As expected, the digestion of EBN with fruit juices and enzymes obviously elevated the free sialic acid content up to 15 g/kg (1.5 %) in HPJ, HPP, and HCJ 4 h, and 18 g/kg (1.8 %) in HAJ and HBM 4 h (Fig. 6).

Enzymatic hydrolysis has been considered a safe method to extract sialic acid in EBN as the compound is stable and less prone for chemical modifications in this process [54]. Food-grade enzymes such as pepsin, pancreatin F, and pepsin-pancreatin F combination were capable of breaking down glycoproteins into glycopeptides and releasing sialic acids located at the terminal of the glycan chains in glycoproteins. Thus, they were applied to increase the free sialic content of EBN and produce hydrolysates with boosted anti-tyrosinase, antiviral, and neuroprotective activities [14,55,56]. When EBN was treated with fruit juices (papaya, pineapple, and cantaloupe juices) and enzymes (papain and bromelain) in our study, the free sialic content was also elevated, which possibly contributed to the improved anti-tyrosinase activity of EBN (Figs. 4 and 6). However, sialic acid was not a sole bioactive compound responsible for this property as EBN hydrolysates by fruit juices inhibited tyrosinase activity more strongly than EBN hydrolysates by respective fruit enzymes despite a similar increase in free sialic content. Distinct bioactive peptides in EBN hydrolysates might also be another factor involved in this difference.

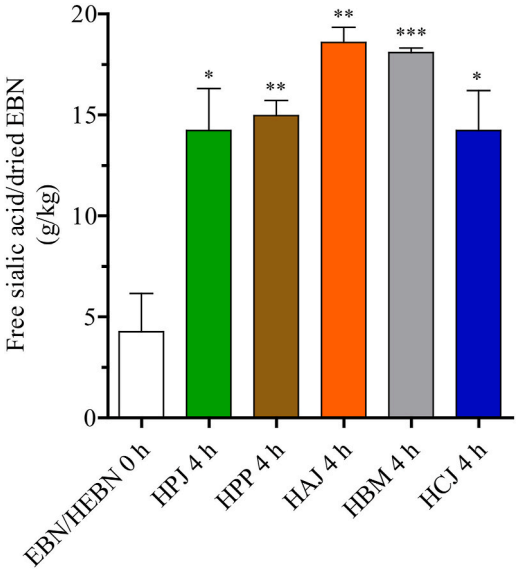

Fig. 6. The free sialic acid content of the EBN hydrolysates by fruit juices, papain, and bromelain. Each data bar is shown as mean ± SD (n = 2). Statistical significance between EBN hydrolysates and unhydrolyzed EBN was calculated by Student's t-test, *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$. HEBN 0 h: mixtures of EBN with fruit juices and enzymes. HPJ 4 h, HAJ 4 h, HCJ 4 h, HPP 4 h, and HBM 4 h: EBN hydrolysates by papaya juice, pineapple juice, cantaloupe juice, papain, and bromelain at 4 h of hydrolysis, respectively.

4. Conclusion

Our study revealed that papaya, pineapple, and cantaloupe juices showing proteolytic activities were able to hydrolyze EBN with effective DH values (about 22 % for papaya juice, 27 % for pineapple juice, and 25 % for cantaloupe juice after 4 h of treatment). EBN hydrolysates by fruit juices displayed higher protein solubility, $ABTS^{\bullet+}$-scavenging, $^{\bullet}OH$-scavenging, and anti-tyrosinase capacities, and free sialic acid contents than unhydrolyzed EBN. Commercial enzymes of plant origin, papain and bromelain, which digested EBN at similar DH values as papaya and pineapple juices increased the amount of free sialic acid as comparably as respective juices. Other nutraceutical effects including protein solubility, $ABTS^{\bullet+}$-scavenging, OH-scavenging, and anti-tyrosinase capacities of EBN were also enhanced by the treatment with papain and bromelain but with less effectiveness than the treatment with respective juices. Noteworthy, the EBN hydrolysates by fruit juices significantly ameliorated the wound-healing process in human fibroblasts whereas this ability was not observed in the EBN hydrolysates by papain and bromelain. Our findings signify the prospect of fruit juices in preparing EBN hydrolysates with enhanced nutraceutical values. Certainly, there are some limitations in the present study which should be taken into account. Although the antioxidant, anti-tyrosinase, and wound-healing abilities of EBN hydrolysates by fruit juices were investigated, responsible bioactive peptides have not yet been identified. Additionally, the evaluation of sensory profiles, consumer acceptability, and reproducibility in the industrial production of the hydrolysates is still lacking. These issues need to be resolved in further investigations.

Data availability statement

Has data associated with your study been deposited into a publicly available repository? No. Data will be made available on request.

CRediT authorship contribution statement

Thi-Phuong Nguyen: Writing – original draft, Methodology, Formal analysis, Data curation. **Quang Thai Le:** Formal analysis, Data curation. **Cong Chinh Bui:** Methodology, Data curation. **Kim Nhung Ta:** Supervision, Methodology. **Khoa Thi Nguyen:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30879.

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