Anti-aging bioactivities of egg white hydrolysates

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Songklanakarin Journal of Science and Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>SJST-2017-0462.R2</td>
</tr>
<tr>
<td>Manuscript Type</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>23-Apr-2018</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Jearranaiprepame, Supisara; Khon Kaen University, Pharmaceutical Technology  
Enmnaisiri, Supak; Khon Kaen University, Pharmaceutical Technology  
Jangpromma, Nisachon; Khon Kaen University, Forensic Science  
Khunkitti, Watcharee; Khon Kaen University, Pharmaceutical Technology |
| Keyword:       | Egg white hydrolysates, Antioxidant peptides, Anti-aging properties, Bioactive proteins |
Original Article

Anti-aging bioactivities of egg white hydrolysates

Supisara Jearranaipreame¹, Nisachon Jangpromma², and Watcharee Khunkitti¹*

¹ Department of Pharmaceutical Technology, Faculty of Pharmaceutical Science, and Biofilm Research Group, Khon Kaen University, Khon Kaen 40002, Thailand

² Protein and Proteomics Research Center for Commercial and Industrial Purposes, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

* Corresponding author, Email address: watkhu@kku.ac.th., watkhu@yahoo.com
Abstract

Egg whites can be readily hydrolyzed to produce small fragments which may be a good source of bioactive peptides. The aim of this study was to investigate anti-aging bioactivities of peptides produced by alkaline hydrolysis. Results demonstrated that neutralized protein/peptides hydrolysates (EWH) at 5 mg/ml showed high antioxidant activity on free radical-scavenging activity at 67.1 ± 1.7 % and inhibited lipid peroxidation at 81.1 ± 2.9 %. Moreover, it possessed high reducing power equivalent to 0.2502 mg of vitamin C. EWH exhibited better antioxidant activity than ovalbumin. In dermal cell culture, EWH increased proliferation and cell migration of keratinocyte HaCat cell and fibroblast NHDF cell and inhibited nitric oxide production of RAW 264.7 indicating its anti-inflammatory action at as low as 0.031 mg/ml. In vivo studies on the prevention of premature skin aging are worth for further investigation.

Keywords: Egg white hydrolysates, Antioxidant peptides, Anti-aging properties, Bioactive proteins

1. Introduction

Skin aging is characterized by a reduction of epidermal thickness, a flattening of the basal membrane, loss of elasticity, irregular keratinization and a decrease in skin lipids. Signs of skin aging are usually noticed as fine lines and wrinkles. After the age of 40, there is a 1-2% annual decrease in collagen and elastin (Travis, Darren & Zimei, 2014). The external factors cause premature aging of skin include oxidative stress, which is triggered by the release of free radicals in the skin mainly by UV rays from sunlight, along with environmental pollutants and smoking. Free radicals are highly reactive molecules containing unpaired electrons that damage skin structures (Ratnam et al., 2006). A number of reports describe the antioxidative properties of
peptides purified from protein hydrolysates, such as those from α- and β-lactalbumin and lecithin-free egg yolk. Egg white proteins also possess antioxidative activities against free radicals (Park, Suzuki, & Lennarz, 2001; DaValos, Miguel, Bartolome, & LaPez-FandiO, 2004; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Tanzadehpanah, Asoodeh, & Chamani, 2012).

Bioactive proteins and peptides have been used in anti-aging cosmeceutical products. These peptides are thought to act by stimulating fibroblast production of collagen or decreasing collagenase breakdown of existing collagen, reducing inflammation, improving cell migration and enhancing wound healing. There are three main categories of cosmeceutical peptides. Firstly, signal peptides which help to increase fibroblast production of collagen or decrease collagenase enzymes e.g. valine-glycine-valine-alanine-proline-glycine peptide, lysine-threonine-threonine-lysine-serine peptides. Secondly, neurotransmitter-affecting peptides which function to reduce muscle contraction and decrease wrinkle size and skin roughness e.g. acetyl hexapeptide-3 (Argireline®), peptapeptide-3 (Vialox®). Thirdly, carrier peptides which function to stabilize and deliver important trace elements necessary for wound healing process, e.g., copper tripeptide complex (Lupo & Cole, 2007; Secchi, 2008).

Egg white proteins are commonly used in the food industry due to their gelling, foaming and emulsifying properties and high nutritional quality. Several studies have demonstrated that bioactivities and functional properties of egg white protein hydrolysates (EWH) are different among methods of hydrolysis (Van der Plancken, Van Loey, & Hendrickx, 2006). For examples, Xu et al. (2007) (Xu, Wang, & Chen, 2007) found that enzymatically derived ovalbumin, which is mainly found in egg white proteins, possessed antioxidant activity through inhibition of superoxide anion, hydroxyl radicals, and lipid peroxidation, in vitro as well as by
elevating the activity of SOD, GSH-Px and CAT indicating tissues protection in mice. Chen et al. (2012) (Chen, Chi, Zhao, & Xu, 2012) demonstrated that antioxidant activity and angiotensin-I converting enzyme inhibitory activity of EWH prepared with trypsin increased as degree of hydrolysis increased. However, few studies on alkali treatment with egg white proteins have been reported. Mine (1997) (Mine, 1997) found that the combination of dry heat and mild alkali treatment of egg white proteins improved their functional properties. Van der Plancken et al. (2005) (Van der Plancken, Van Loey, & Hendrickx, 2005) demonstrated that the combination of pressure and alkali treatment of egg white solutions appeared to decrease protein solubility and total sulfhydryl content, whereas at high pressure and temperature, some of the SH groups remained unoxidized. However, their bioactivities were not reported. Therefore, the aim of this study was to investigate anti-aging bioactivities of egg white alkali hydrolysates treated with high pressure and temperature.

2. Materials and Methods

Materials

Hen eggs were obtained from Polwittaya Farm (Khon Kaen, Thailand). Potassium hydroxide was purchased from RCI Labscan (Bangkok, Thailand). Hydrochloric acid, fuming 37%, was purchased from VWR International (PA, USA). Trolox, glutathione (GSH) and ovalbumin (OVA) were purchased from Sigma-Aldrich (MO, USA). Vitamin E acetate was purchased from Namsiang Co., Ltd. (Bangkok, Thailand). Vitamin C was purchased from S. Tong Chemicals Co., Ltd. (Nonthaburi, Thailand). Other chemicals used in experiment tests were analytical grade. DPPH, 2,2'-Azo-bis-amidinopropane (ABAP), ferrous chloride, trichloroacetic acid, ammonium thiocyanate, potassium ferricyanide, ferric chloride, tris hydrochloride, β-
mercaptopethanol, sodium dodecyl sulfate, Dulbecco’s Modified Eagle’s medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), antibiotic-antimycotic, L-glutamine and PrestoBlue® were purchased from Sigma-Aldrich (MO, USA). TEMED was purchased from Thermo Fisher Scientific (MA, USA).

Egg white hydrolysates

Methods of egg white hydrolysates (EWH) preparation

Egg whites (EW) were separated from hen eggs and mixed with 0.4 N KOH at a ratio of 1:3. The mixture was hydrolyzed in water bath at 55°C for 2 h with continuous stirring and autoclaved at 121°C, 15 psi for 2 h. Then, the solution was filtered through 5 layers of gauze and neutralized (pH=7) using hydrochloric acid fuming 37%. The hydrolysate was lyophilized and stored at -40°C.

Percent yield

Egg white hydrolysate (EWH) solution was weighed in pre-weighed jars. Then, the samples were lyophilized and weighed as dried powder. Percent yield of dried EWH was calculated by equation (1).

\[
\% \text{ yield} = \frac{\text{Weight of lyophilized EWH}}{\text{Weight of fresh EW}} \times 100
\] (1),

Characterization of EWH

Determination of total protein

A stock solution of bovine serum albumin (BSA) as a standard protein at a concentration of 1000 µg/ml was prepared and diluted with deionized water (DI) in a range of 10-500 µg/ml. A 20 µl of
the samples were pipetted into 96-well plate. Then, 180 µl of Bradford dry reagent were added and mixed well. The mixtures were kept at room temperature (25°C) for 5 min. The experiment was performed in triplicate. The absorbance of the test samples was measured using a UV-Visible spectrophotometer at 595 nm. The absorbance was plotted versus the concentrations of BSA solutions to make a standard curve of BSA. The EWH samples were prepared in the same manners as the standards and measured the absorptions at 595 nm. The total protein of EWH was then calculated (Bradford, 1976).

**Determination of molecular weight using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the components of the EWH. Amersham Rainbow Markers (3.5-38 kDa) RPN755E (GE Healthcare Life Science, Sweden) were used to compare the hydrolysates. Briefly, 15% (v/w) acrylamide separating gel and 4% (v/w) acrylamide stacking gel were prepared. The hydrolysate sample at a protein concentration of 30 µg/µl was pipetted (10 µl) into 1.5 ml test tube and mixed with a 10 µl of 2X solubilizing dye with β-mercaptoethanol. Then, the mixture was heated at 100°C for 5 min. The samples were pipetted into the gel sheet and run at 140 V for about 40 min to separate protein bands. After the electrophoresis process finished, the gel sheet was stained with Coomassie brilliant blue G-250 (CBB) staining solution. (Wang, Su, Jia, & Jin, 2013).

**Amino acids analysis**
The EWH was prepared by EZ:faast–Amino acid analysis of protein hydrolysates by LC-MS method. Sample volumes of 1 µl were injected into the LC mounted with EZ:faast AAA-MS column 250 x 3.0 mm and eluted at 35°C with flow rate of 0.5 ml/min. The mobile phase A was water containing 10 mM ammonium formate, and B was methanol containing 10 mM ammonium formate. The gradient consisted of 68% B for 13 min, with linear increase to 83% B in 13 min, and re-equilibration at 68% B until the end of run (23 min). The mass spectrum was run in the positive ion mode scanning range from 100-600 m/z. The APCI ionization chamber temperature was 450°C.

Bioactivities of EWH

Antioxidant activities tests

*DPPH radical scavenging assay*

Radical scavenging activity of EWH was measured in term of DPPH radical-scavenging activity. Each 100 µl of sample either hydrolysates or standards (Trolox, vitamin E acetate, Glutathione, and vitamin C) were pipetted into the first column of 96 well-plate and diluted 2-fold with DI water or 80% (v/v) ethanol. The 0.004 M 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH) was prepared by dissolving in 80% (v/v) ethanol and then 50 µl were added to each well, except blank wells (samples without 0.004 M DPPH). The samples were mixed and then kept at room temperature in the dark for 25 min. The color reduction of the DPPH substrate was measured by UV-Visible spectrophotometer at wavelength of 517 nm and calculated as DPPH radical-scavenging activity (%) using equation (2) (Veerapan, & Khunkitti, 2011):

\[
\text{DPPH radical – scavenging activity} \, (\%) = \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \quad (2)
\]
Abs\textsubscript{control} = Absorbance of solvent with 0.004 M DPPH

Abs\textsubscript{sample} = Absorbance of sample with 0.004 M DPPH

Abs\textsubscript{blank} = Absorbance of sample without 0.004 M DPPH

**Linoleic acid peroxidation inhibition (LPO)**

A stock solution of linoleic acid (50 µl/ml in 80% (v/v) ethanol) was prepared. Then 50 µl of the stock solution was mixed with 50 µl of the standard solutions (Trolox, vitamin E acetate, Glutathione, and vitamin C) and EWH in the 1.5 ml test tube. A 10 µl of 2,2'-Azo-bis-amidinopropane (ABAP) was added into each tube, except the blank tubes (sample without 0.07 M ABAP). Then, a 150 µl of 20% (v/v) acetic acid was pipetted into each tube. All test tubes were vortexed and incubated at 70°C for 1 h. After that, 20 µl of the reaction mixtures were mixed with 160 µl of 75% (v/v) ethanol in a 96 well-plate. Then 10 µl of 15% (w/v) ammonium thiocyanate and 0.05 M ferrous chloride were added sequentially. The absorbances were measured by a UV-Visible spectrophotometer at 500 nm and values of % lipid peroxidation inhibition were calculated using equation (3) (Ajibola, Fashakin, Fagbemi, & Aluko, 2011):

\[
\%\text{Lipid peroxidation inhibition} = \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100
\]  

\text{(3)},

Abs\textsubscript{control} = Absorbance of solvent (water or ethanol) with 0.07 M ABAP

Abs\textsubscript{sample} = Absorbance of standards and EWH with 0.07 M ABAP

Abs\textsubscript{blank} = Absorbance of standards and EWH without 0.07 M ABAP

**Reducing power assay**

For Proof Read only
All hydrolysates and the reference standards (Trolox, vitamin E acetate, Glutathione, and vitamin C) were dissolved in DI water or 80% (v/v) ethanol as solvent at difference concentrations. Then 250 µl of each preparation was mixed with 250 µl of 0.2 M sodium phosphate buffer pH 6.6. and 250 µl of 1% (w/v) potassium ferricyanide. The samples were mixed and incubated at 50°C for 20 min. Then 250 µl of 10% (w/v) trichloroacetic acid were added. The solutions were mixed together and incubated at 25°C for 10 min. All preparations were centrifuged at 800 rpm for 10 min. The supernatants (30 µl) were diluted with distilled water (160 µl) in a 96 well-plate and then 10 µl of 0.1% (w/v) ferric chloride were added and allowed to stand for 10 min at room temperature. The absorbance was measured using a UV-Visible spectrophotometer at 700 nm and calculated as vitamin C equivalent (mg) using a vitamin C standard curve (Oyaizu, 1986).

The effect of EHW on dermal cell culture

Cell cultures

Normal human dermal fibroblast cells (NHDF cell) and human immortalized keratinocyte cell line (HaCat cell)

Normal human dermal fibroblast cells (NHDF cell) and human immortalized keratinocyte cell line (HaCat cell) were used in this study. NHDF cell were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-aldrich, USA), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotic and 1% (v/v) L-glutamine, whereas HaCat cell was cultured in DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. All cells types were seeded in 75 cm² T-flask and cultivated at 37°C with 5% CO²/95% relative
humidity (RH). Cells were subcultured with 0.25 % (w/v) Trypsin-EDTA when they reached about 80%-90% confluence (Sayes et al., 2006).

**Murine macrophage cell line RAW 264.7 (RAW 264.7)**

Murine macrophage cell line RAW 264.7 was cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Sigma-aldrich, USA), supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. The cells were seeded in 75 cm² T-flask and cultivated at 37°C with 5% CO₂/95% relative humidity (RH). Cells were subcultured using a cell scraper when they reached around 80%-90% confluence (Sayes et al., 2006).

**Anti-inflammation activity using nitric oxide inhibition assay**

The nitric oxide inhibition assay was determined in RAW 264.7. The cells were seeded into a sterile 96 well-plate at 5 x 10⁴ cells/well and cultured at 37°C with 5% CO₂/95% relative humidity (RH) for 24 h. Then, the medium was removed and cells were washed twice with phosphate buffer solution pH 7.4. 200 µl of the samples and standard (diclofenac sodium) in RPMI 1640 medium containing 100 ng/ml of Lipopolysaccharides (LPS) were pipetted into cell culture plate. Controls received only fresh RPMI 1640. then the plate was incubated at 37°C with 5% CO₂/95% relative humidity (RH) for 24 h. To determine nitric oxide reduction, the 100 µl of the sample was mixed with 100 µl Griess reagent containing 1% (w/w) sulfanilamide and 0.1% (w/w) N-1-[naphthyl] ethylenediamine dihydrochloride in 2.5% (w/w) H₃PO₄. The absorbance of the solution was determined after 10 min by Varioskan flash microplate reader (Thermo Fisher, Finland) at 550 nm. The results were reported as % nitric oxide reduction which was calculated by the equation (6) (Hernández-Ledesma, Hsieh, & de Lumen, 2009).
% nitric oxide reduction = \frac{Abs_{control}- (Abs_{sample}- Abs_{blank})}{Abs_{control}} \times 100 \quad (6)

Abs_{control} = \text{Absorbance of cell treated with medium with LPS}

Abs_{sample} = \text{Absorbance of cell treated with EWH and medium with LPS.}

Abs_{blank} = \text{Absorbance of cells treated with EWH and medium without LPS.}

Scratch-wound assay

The NHDF cells and HaCat cells were seeded into 6-well plate at density of $1.25 \times 10^5$ cells/well and $3.5 \times 10^5$ cells/well, respectively. The plates were incubated at $37^\circ$C with 5% CO$_2$/95% relative humidity (RH) for 24 h. After incubation, the adherent cell layer was scratched to 3 vertical lines with a sterile yellow pipette tip (200 µl) and then medium was removed. Cellular debris was removed by washing with phosphate buffer solution, pH 7.4. The cells were treated with 2 ml of the medium containing 0.031, 0.125, 0.5 and 2 mg/ml of EWH and standard OVA with concentration of 0.031 mg/ml which prepared as the same method as cell viability test. The medium was used as control. The cells were incubated (at $37^\circ$C with 5% CO$_2$/95% relative humidity (RH) for 0, 12 and 24 h) and then the imaging of the scratch area was carried out at two different points using an inverted fluorescence microscope (Carl Zeiss Microscopy, Germany) at 0 h (just after scratching cells) and at 12 h and 24 h after incubation with EWH, OVA and control. Data were analyzed with the Image Pro Plus 7.0 program (Media Cyberneties, USA) in order to determine the width of the scratch and thus to calculate the rate of migration of cells by the following equation (5) (Z. Wang, Wang, Farhangfar, Zimmer, & Zhang, 2012):
\[
\% \text{ Migration rate} = \left( \frac{\text{Area}_0 - \text{Area}_n}{\text{Area}_0} \right) \times 100 \quad (5)
\]

\text{Area}_0 = \text{wound areas at time 0 h}

\text{Area}_n = \text{wound areas at 12, and 24 h}

\textbf{Cell viability assay}

NHDF cell and HaCat cell were seeded in a 96-well sterile plate with flattened bottom at \(1 \times 10^5\) cells/well and \(2 \times 10^5\) cells/well, respectively. Both cells preparations were cultivated overnight at 37°C with 5% CO\(_2\)/95% relative humidity (RH). Then, cells were washed with phosphate buffer solution pH 7.4 (100 µl). The EWH and standard OVA prepared in a range of 0.031-10 mg/ml with DMEM supplemented with 10% (v/v) FBS. Each 200 µl of samples either EWH or standards were added and the medium was used as control.

RAW 264.7 cell was seeded in a 96-well sterile plate with flattened bottom at \(7.5 \times 10^4\) cells/well and cultivated overnight at 37°C with 5% CO\(_2\)/95% relative humidity (RH). Then, the cells were washed with phosphate buffer solution pH 7.4. Each 200 µl of samples were added and the medium was used as a control.

The cell viability was measured at 0, 6, 12 and 24 h, respectively. At the end of each incubation time, all solutions were removed and the cell viability was measured using Prestoblue\textsuperscript{®} mixture (50 µl) (Sigma-aldrich, USA) which was prepared as 1:9 parts of Prestoblue\textsuperscript{®}: cell medium. The plate was incubated at 37°C with 5% CO\(_2\)/95% relative humidity (RH) for 90 min. After reaction, the emission of the mixture was determined by using Verioskan flash microplate reader (Thermo Fisher, Finland) at wavelengths 560/590 nm. The results were reported as % cell viability which was calculated using equation (4) (Fischer, Li, Ahlemeyer, Kriegstein, & Kissel, 2003):
% cell viability = \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (4),

Abs_{control} = \text{Absorbance of cells treated with medium at 0 h}

Abs_{sample} = \text{Absorbance of cells treated with EWH at the time intervals}

Statistical analysis

All experiments were performed in triplicates. The results were expressed as the mean ± SD. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). The significant differences among test samples were analyzed by ANOVA. Tukey post hoc test was used for multiple comparison analysis. A p < 0.05 was considered statistically significant.

3. Results

The percent yield and total protein of EWH was 22.40 ± 0.24, with BSA equivalent of 0.4695 ± 0.7304 g. The EW without hydrolysis had molecular weights that clustered in the 13-14 kDa and about 15-38 kDa range (Figure 1); EWH displayed molecular weights in the range below about 3.5 kDa. The lower band intensities could be a result of significant migration of very low molecular weight species to the lower electrode buffer.

The amino acid profiles of EWH are shown in Table 1. Neutralized EWH contained many types of amino acid, with the highest amounts for aspartic acid (Asp, which was a negatively charged carboxyl side chain at pH 7), lysine (Lys), leucine (Leu) and alanine (Ala, which contains protonated amine groups at pH 7).
Antioxidant activities of EWH were determined using three methods; Free radical scavenging capacity assay (DPPH), Linoleic acid peroxidation inhibition assay (LPO) and Reducing power assay. In the DPPH assay, EWH at 5 mg/ml possessed DPPH radical scavenging activity with % DPPH radical-scavenging activity at 67.1 %. Moreover, it exhibited significantly higher values than OVA, which was high protein in egg white, and vitamin E acetate (p<0.0001) (Table 2).

In linoleic acid peroxidation inhibition assay (LPO), EWH showed activities in lipid peroxidation inhibition at 81.1 % (Table 2). However, it seems that these EWH possessed significantly more inhibition activity than OVA, vitamin E acetate and glutathione, respectively (p<0.05).

Reducing power activities of EWH are substantial. At a concentration of 50 mg/ml of hydrolysate, EWH exhibited high reducing power with abilities equivalent to vitamin C 0.2502 mg. Furthermore, the reducing power of EWH was significantly higher than 50 mg/ml of OVA, 0.24 mg/ml of vitamin E acetate, 0.15 mg/ml of GSH and 0.125 mg/ml of trolox (p<0.05) (Table 2).

Figure 2 shows the effect of EWH on nitric oxide production. The lowest concentration of EWH (0.031 mg/ml) exhibited the highest % nitric oxide reduction at 20.87 ± 5.02 % when compared with control medium and it was not significantly different from 0.1 mg/ml diclofenac sodium. It showed that the activity decreased when the concentration was increased. This study showed that EWH enhanced migration of keratinocytes. At 12 h of incubation, EWH at 0.031 mg/ml and 0.125 mg/ml exhibited a significantly higher percent keratinocyte migration of 37.83% and 36.66%, compared to the control medium (26.47%) (p<0.05). Furthermore, it significantly increased cell migration at 52.32% and 49.74%, greater than the control (38.08%) (p<0.05) at 24
h of incubation (Table 3 and Table 4). However, it should be noted that the percent fibroblast migration of EWH at the same concentrations showed significantly faster than the control at 12 h but slower rate was found at 24 h with no significant difference (Table 3).

PrestoBlue® Cell Viability Reagent was used to measure cell proliferation. It was a ready-to-use cell permeable resazurin-based solution. When added to cells, the PrestoBlue® reagent is reduced by enzymes in the mitochondria of viable cells and turns into the red-colored resorufin, whose fluorescence emission at 560/590 nm was determined. The cell proliferation was evaluated in NHDF cell, HaCat cell and RAW 264.7.

In RAW 264.7, at 12 and 24 h, although the viability of RAW 264.7 exposed with EWH at all concentrations was not significantly different from control and 0.031 mg/ml OVA, it was found that the viability of RAW 264.7 treated with 0.031 mg/ml EWH was significantly less than that of the cells treated with 0.125-0.5 mg/ml EWH. The viability of RAW 264.7 tended to increase as the concentrations of EWH increased. (Figure 3). Moreover, this study also demonstrated that EWH stimulates fibroblast proliferation. At 12 h of incubation, the EWH at 0.031 mg/ml significantly increased cell viability up to 111.23%, which was greater than the control (105.10%) (p<0.05) (Figure 4). Moreover, EWH at 0.031 mg/ml promoted keratinocyte proliferation (Figure 5). At 6 h of incubation, it had significantly higher cell viability (120.46 %) than the control (104.59 %). At 24 h of incubation, EWH at 0.031 mg/ml and 0.125 mg/ml were significantly increased cell viability at 191.84 % and 166.64%, respectively which were greater than the control (166.27%) (p <0.05). However, Keratinocyte proliferation of EWH was not significantly different from that of OVA.
4. Discussion

In this study, it was found that antioxidant activities of EWH were greater than OVA. EWH possessed moderate free radical scavenging, high lipid peroxidation inhibition and reducing power activities in comparison with trolox, vitamin C and glutathione, respectively. Antioxidant activity of EWH might be due to low molecular weight polypeptides (<3.5 kDa) and amino acids components. Several studies demonstrated that the antioxidant abilities of peptides depend on their molecular weight, types of amino acid and amino acids sequence in peptides chains. The small peptides can act as antioxidant agents better than the long chain peptides (Cho et al., 2014). Although the detailed nature of the peptide and amino acid composition of the hydrolysates have not been studied, the amino acid profile of the hydrolysate shows high amounts of electrically charged side chains amino acid (such as, aspartic acid, glutamic acid, and lysine) and hydrophobic amino acid contents (such as, leucine, valine, and alanine). Some of amino acid in hydrolysate including tyrosine (Tyr), histidine (His), glutamic acid (Glu), and leucine (Leu) could donated hydrogen or electron to DPPH free radical (Abeyrathne, Lee, & Ahn, 2013). Accordingly, the hydrolysate components act as radical scavenging compounds. Moreover, this hydrolysate inhibited lipid peroxidation activities. Most of amino acid residues in EWH contains of hydrophobic amino acid such as alanine (Ala), leucine (Leu), tyrosine (Tyr), valine (Val), and phenylalanine (Phe). The hydrophobic amino acid are probably important for protecting hydrophobic substances such as lipids (Zhuang, Zhao, & Li, 2009). In particular, hydrophobic peptides in hydrolysates may donate protons to lipid radicals. In contrast, OVA contain large protein molecules which make them harder to insert its chains into the lipid cell membrane. As a result, it could not inhibit lipid radicals (Chen & Chi, 2012; Memarpour-Yazdi, Asoodeh, & Chamani, 2012). Furthermore, EWH, which contained high amount of acidic and basic amino
acid, such as aspartic acid (Asp), lysine (Lys), glutamic acid (Glu) and arginine (Arg) in peptides chains, possessed good reducing power. They may act as metal chelators through binding the side chains and N-terminal and C-terminal groups (Abeyrathne et al., 2013).

RAW 264.7, a murine macrophage cell, was activated by LPS to produce a large quantity of nitric oxide (NO) which is the major inflammatory mediators and can lead to induce damage cells and tissues around the wound area. In this study, a range of EWH concentrations (0.031-0.5 mg/ml) had no cytotoxic effects on RAW 264.7. The proliferation of RAW 264.7 exposed with EWH in a range of 0.031-0.5 mg/ml was increased in a dose-dependent manner. EWH at low concentration (0.031 mg/ml) appeared to have anti-inflammatory action in some extent. However, nitric oxide inhibition was decreased as EWH concentrations increased. Napoli et al. (2013) reported that the effect of NO production on cellular process depends on its concentration and on the presence of other free radicals. This results showed that NO inhibition at low concentration appears to exert a direct effect on cell proliferation and survival whereas at higher EWH, some parts of EWH might inhibit NO production and the remaining EWH might nurture the RAW 264.7 proliferation and produce NO during proliferation process resulting in lowering NO inhibition. However, anti-inflammatory action of EWH could also occur by inhibition of other inflammatory pathways, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-1β, interferon gamma (IFN-γ), and IL-17 and cytokines, in the inflammatory pathway. Several studies had shown that egg whites possess anti-inflammation properties. For examples, Choi et al. (2013) (Choi, Park, Yoo, & Choi, 2013) found that egg white-chalcanthite can inhibited nitric oxide and prostaglandin E2 in LPS-stimulated BV2 microglia. It also attenuated the expression of nitric oxide synthase, cyclooxygenase-2 and pro-inflammatory cytokines such as, IL-1β and TNF-α. (Zhao et al. ( 2017) found that simulated gastrointestinal
digest from preserved egg white can inhibit the secretion of interleukin (IL)-8, reduce TNF-α in a
ccentration-dependent manner.

It should be noted that keratinocyte and fibroblast migration and in the proliferation assay were
decrease as the EWH concentration increased. This is probably due to the formation of soluble
salts from the neutralization process in the EWH solutions at high concentrations interfering with
the electrolyte balance of cells, leading hypertonicity resulting in cell dead (Robbins, 1970). In
addition, EWH at low concentration motivated fibroblast and keratinocyte proliferation and
migration. This finding suggested that EWH at low concentration might have a wound healing
effect. However, fibroblast migration was significantly found at 12 h and slower migrated at 24
h. It might be possible that EWH may contain essential amino acids which are important for cell
activities and amino acid sequences in the various peptide chains similar to cytokines and growth
factors, thus providing cell nutrients (Ye et al., 2016). According to a review on wound healing
process (Gonzalez et al. 2016), these findings suggested that EWH at low concentration might
involve in controlling inflammation phase of wound healing. Then, fibroblasts located on the
skin edge begin to proliferating and probably synthesize and secret keratinocytes growth factor
which stimulate neighboring keratinocytes to migrate in the wound area, proliferate, and
differentiate in the epidermis in proliferative phase.

5. Conclusions

EWH prepared by alkaline hydrolysis under high pressure and temperature appeared to have
multifunctional antiaging cosmetic activities, such as, free radicals scavenging, inhibited lipid
peroxidation and reducing power activities. Moreover, EWH at an optimal concentration may
help to improve wound healing process. Therefore, EWH may be a good candidate as an
antiaging ingredient. However, *in vivo* studies and clinical trials are necessary to establish the antiaging properties and allow for development of cosmeceutical products.

References


Figure 1 SDS-PAGE bands of Amersham Rainbow Markers, EW and EWH

Figure 2 Nitric oxide inhibition abilities of standard diclofenac sodium, OVA and EWH in the RAW 264.7

*p-value < 0.05 compared with diclofenac sodium (standard)
*p-value < 0.05 compared with OVA
Figure 3 Cell proliferation activities of EWH, OVA and control medium in RAW 264.7
The different alphabets (a-b) indicated significant differences (p<0.05)

Figure 4 Cell proliferation activities of EWH, OVA and control medium in NHDF cell
**p-value < 0.01, *p-value < 0.05 compared with control medium
a p-value < 0.05 compared with OVA
Figure 5 Cell proliferation activities of EWH, OVA and control medium in HaCat cell

**p-value < 0.01, *p-value < 0.05 compared with control medium

* p-value < 0.05 compared with OVA
### Table 1: Amino acid profiles of neutralized EWH

<table>
<thead>
<tr>
<th>Types of amino acid</th>
<th>Neutralized EWH (g/100g of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>9.38</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.78</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>19.24</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.00</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8.93</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.10</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.2</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.00</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.75</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.86</td>
</tr>
<tr>
<td>Lysine</td>
<td>16.49</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.42</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.78</td>
</tr>
<tr>
<td>Proline</td>
<td>8.20</td>
</tr>
<tr>
<td>Serine</td>
<td>2.23</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.84</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.55</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.06</td>
</tr>
<tr>
<td>Valine</td>
<td>8.44</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.00</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.00</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.87</td>
</tr>
</tbody>
</table>
Table 2 Antioxidant activities of EWH, OVA and standards

<table>
<thead>
<tr>
<th>Samples</th>
<th>% DPPH radical-scavenging activity (at 5 mg/mL)</th>
<th>% Linoleic acid peroxidation inhibition (at 5 mg/mL)</th>
<th>Reducing power* equivalent to Vitamin C (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>85.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Trolox</td>
<td>95.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102.5 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0884 ± 0.0052&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>75.4 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>65.8 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0927 ± 0.0019&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>42.2 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>58.7 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0126 ± 0.0034&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ovalbumin (OVA)</td>
<td>22.8 ± 2.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>66.4 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0225 ± 0.0007&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EWH</td>
<td>67.1 ± 1.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td>81.1 ± 2.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2502 ± 0.0174&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with different alphabets (a-f) in each column indicated significant differences (p<0.05)

*Concentration of each substance in reducing power assay: trolox 0.125 mg/mL; GSH 0.15 mg/mL; vitamin E acetate 0.24 mg/mL; OVA 50 mg/mL and EWH 50 mg/mL.
Table 3 Percent migration abilities of EWH, OVA and control medium in fibroblast and keratinocyte cell

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration (mg/ml)</th>
<th>% Fibroblast migration</th>
<th>% Keratinocyte migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>EWH 0.031 mg/ml</td>
<td>27.39 ± 0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.83 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EWH 0.125 mg/ml</td>
<td>25.04 ± 2.61</td>
<td>36.66 ± 2.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EWH 0.5 mg/ml</td>
<td>23.70 ± 1.56</td>
<td>27.55 ± 4.04</td>
</tr>
<tr>
<td></td>
<td>EWH 2 mg/ml</td>
<td>24.55 ± 1.22</td>
<td>25.97 ± 2.12</td>
</tr>
<tr>
<td></td>
<td>OVA 0.031 mg/ml</td>
<td>23.22 ± 1.60</td>
<td>23.45 ± 3.53</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>23.79 ± 1.40</td>
<td>26.47 ± 3.93</td>
</tr>
<tr>
<td>24 h</td>
<td>EWH 0.031 mg/ml</td>
<td>52.11 ± 0.63</td>
<td>52.32 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EWH 0.125 mg/ml</td>
<td>49.11 ± 3.00</td>
<td>49.74 ± 1.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EWH 0.5 mg/ml</td>
<td>48.62 ± 0.99</td>
<td>39.18 ± 4.54</td>
</tr>
<tr>
<td></td>
<td>EWH 2 mg/ml</td>
<td>50.00 ± 2.09</td>
<td>38.00 ± 1.92</td>
</tr>
<tr>
<td></td>
<td>OVA 0.031 mg/ml</td>
<td>50.96 ± 1.67</td>
<td>34.92 ± 3.65</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>49.11 ± 3.81</td>
<td>38.08 ± 6.11</td>
</tr>
</tbody>
</table>

<sup>**</sup>p-value < 0.01, <sup>*</sup>p-value < 0.05 compared with control medium
<sup>a</sup>p-value < 0.05 compared with OVA
Table 4 Cell migration of EWH, OVA and control medium in keratinocyte in different time intervals

<table>
<thead>
<tr>
<th>Samples</th>
<th>0 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EWH 0.031 mg/mL</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>EWH 0.125 mg/mL</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>EWH 0.5 mg/mL</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>EWH 2 mg/mL</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>OVA 0.031 mg/mL</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Control</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>