1.0 INTRODUCTION

Photoaging is the phenomenon of skin inflammation and connective tissue damage induced by UV radiation [1]. Among the UV spectrums, UVB (290-320 nm) is the main cause of photoaging. UVB spectrum may penetrate into the dermal layer of skin tissue [2]. The penetration induces oxidative stress and increase
the production of reactive oxygen species (ROS). ROS will activate the mechanism of DNA damage and inflammation genes, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Several enzymes, such as collagen degrading-matrix metalloproteinase (MMP) 1, MMP-2 and MMP-9 are activated along this reaction [3]. This phenomenon, also known as dermatoheliosis, has several esthetic effects, such as skin pigmentation and wrinkle [4].

The cumulative effect of photoaging may lead into major problems of health, initiation of tumor, and increase skin cancer risk [5, 6]. Many researches have been conducted to reduce the skin inflammation and formulating skin protection. Recently, anti-photoaging supplement extracted from Pangasius hypophthalmus was developed [7]. This supplement contains tripeptide collagen which has potential activity to enhance the skin elasticity. It is because of potential peptide which acts as antioxidant. Thus, ROS can be reduced significantly. Moreover, oral supplementation of the peptide reduces formation of the abnormal elastic fiber.

Current trends in cosmeceuticals bring out the potential fermented product as skin whitening and rejuvenating. The most popular fermented product that has been used widely is kefir, a fermented milk product. A total of 236 isolated peptides have been identified from kefir and 16 of them showed the potency as bioactive compound that role as anti-aging agents [8]. Tempeh is one of potential fermented product from Indonesia made from soybean. The fermentation of soybean during tempeh production generates peptides and amino acids. Essential amino acids weight generated from this process was 1.70 g/ 100 g dry matter, respectively [7]. Moreover, tempeh has potency as anti-photoaging agents, because it mainly contained of peptide, oligopeptide, and other nitrogen derivative compounds. It also contains many phytochemicals and isoflavone, such as genistein and daidzein [9]. However, screening of tempeh as potential anti-photoaging agents is lacking. The purpose of this study was to investigate soybean and black soybean tempeh peptides effectiveness to reduce photoaging in mice using matrix metalloproteinases and inflammation biomarkers.

2.0 METHODOLOGY

2.1 Materials

Natrium hydroxide (NaOH) were provided by Sigma (St. Louis, Missouri, USA). All the water used had been distilled to aquadestusing GFL Water Distillation Unit 2008 (GFL, Burgwedel, Germany). The RNA extraction reagent GENEcoli™ was purchased from Geneaid (New Taipei City, Taiwan) while cDNA synthesis reagent ReverTra Ace® qPCR RT Master Mix with gDNA remover and THUNDERBIRD® SYBR® qPCR Mix were provided by Toyobo (Osaka, Japan). All of the primers were synthesized by Integrated DNA Technologies (Singapore).

2.2 Preparation of Peptide Extracts from Soybean and Black Soybean Tempeh

Soybean and black soybean tempeh were collected from Tjah Dampit, a traditional organic tempeh industry in Yogyakarta (Indonesia). A total of 250 g samples were cut and freeze-dried using Martin Christ Alpha 1-2 LDPlus Freeze Dryer (Christ, Osterode, Germany) for 40 hours until become powder. Peptide extraction was done according to previous study with modifications in hydrolytic enzyme [10]. Tempeh powder was diluted with aquadest. The pH was adjusted to 8.0 using 2.5 M NaOH. Enzyplex® powder was added until the concentration reach 1% w/v in the mixture. The mixture was incubated in WiseBath waterbath shaker (Daihan, Wonju, South Korea) at 75 rpm in 37 °C for 30 minutes. After the incubation, the mixture was centrifuged at 4 °C, 3000 ×g for 30 minutes. The supernatant was filtered and dried by lyophilization using freeze dryer. The samples were stored at -20 °C for further analysis. Verification of peptide was done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 15% sample and 18% gel concentration. Peptide molecular weight was determined based on Spectra™ Multicolor Low Range Protein Ladder (ThermoFisher, Massachusetts, USA) as marker. Tempeh peptide extracts had molecular weights of 11.2 and 12.6 kDa (data not shown).

2.3 Identification of Amino Acid Composition in Peptide Extracts Using Ultra Performance Liquid Chromatography

The identification procedure was performed according to Acquity UPLC H-Class and H-Class Bio Amino Acid Analysis System Guide revision B developed by Waters (Milford, MA, USA). A standard solution of mixed amino acid was homogenized with internal standard AABA (1:1) in 920 µL aquabidest. The solution was added with Acc-Q-Fluor Borate and reagent fluor A. After incubation for 1 minute at room temperature, the solution was incubated at 55 °C for 10 minutes.

Derivatization method of peptide for UPLC and elution condition were performed as described previously [11]. Briefly, peptide samples [0.1 g] were hydrolyzed with 1 mL 6N HCl (1:10) at 110 °C for 22 hours. The samples were diluted with aquabidest and then filtered through 0.45 µm syringe filter (Waters, Milford, MA, USA). Acc-Q-Fluor Borate and reagent fluor A were added to the solution and incubated for 1 minute at room temperature before incubated at 55 °C for 10 minutes. The UPLC method was developed for the identification of amino acid composition using AccQ-Tag Ultra C18 Column (2.1 x 100 mm) (Waters, Milford, MA, USA). The flow rate was set at 0.7 mL/min, injection volume was 1 µL, column temperature was set at 49 °C and detection
was carried out with photodiode array detector at 260 nm. The mobile phase consisted of (A) acetonitrile (100% AccQ Tag Ultra Eluent A) and (B) formic acid (90:10 HPLC-grade water:AccQ Tag Ultra Eluent B). This system used gradient elution mode as follows: 0-0.54 min, 0.1%-99.9%B; 5.74 min, 9.1%-90.9%B; 7.74 min, 21.2%-78.8%B; 8.04 min 59.6%-40.4%B; 8.70-10 min, 99.9% A-0.1%B.

2.4 Animals Experiment

The in vivo study was conducted at VStem Animal Facility (Bogor, Indonesia) and the protocol was approved by the Animal Welfare Supervision Commission and Use of Research Animals (No. VS.02-17-IR). A total of 28 male Deutschland, Denken, and Yonken (ddY) mice aged 6 weeks with body weight 25±1 g were divided into seven groups, each contained four mice: negative control (NC) and positive control (PC) without supplementation, peptide control (SP) supplemented with commercial cosmeceuticals, the remaining four supplemented with peptide extracts for 28 days (Table 1). The mice were adapted to the laboratory environment 7 days prior to the treatment and fed with standard diet. The dorsal areas of the mice were shaved to give hairless condition. The mice were housed in group in sanitary ventilated animal rooms under controlled temperature 25±1°C, 60% relative humidity, and fed with standard feed.

The dorsal skin specimens (approximately 1 cm²) were fixed in 4% buffered neutral formalin for at least 24 h and stained using hematoxylin and eosin method [13]. Skin histology changes, including epidermis thickening and formation of dermal junction were observed by light microscopy at 200× and 400× magnification using nominal scoring method [14].

2.6 Determination of Photoaging-related Genes Expressions Using Quantitative Polymerase Chain Reaction

Analysis of qPCR was used to measure the expressions of matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) genes. RNA from the dorsal skin was isolated using GENEzo™ according to the manufacturer’s protocols. cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix with gDNA remover according to the protocols. qRT-PCR was carried out using THUNDERBIRD® SYBR® qPCR Mix performed on iQ5 Multicolor Real Time PCR (BioRad, California, USA) with beta-actin as a reference gene. Primer sequences were listed in (Table 2).

Table 2 Oligonucleotide primer sequences

| Gene | Forward | Reverse | Size (bp) |
|------|---------|---------|-----------|
| MMP-1 | GGGAGCCAGG | TIGGCCTAGCAT | 245 |
| MMP-3 | GGCACAAACTCT | TCTTCGGGAGCA | 217 |
| MMP-9 | AAAGGACGCT | TTAGCCAGAA | 362 |
| COX-2 | CCTCGGAGGCA | CAGGGAG | 252 |
| iNOS | GTGGAACGCAG | GGGTGTCGAG | 355 |
| β-actin | AGGGAAATCGT | TAGGTGTGGCA | 278 |

2.7 Statistical Analysis

All data are presented as the means ± standard deviation from three independent experiments. Data were evaluated using the one way-analysis of variance test and Tukey post-test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, USA). The difference between the fold change (double delta CT) means was considered significant when P ≤ 0.05.

3.0 RESULTS AND DISCUSSION

3.1 Amino Acid Composition in Peptide extracts from Soybean and Black Soybean Tempeh

Peptide cosmeceuticals has been developed from many resources, mostly derived from fish collagen...
such as from silver carp and pangas or tilapia gelatin peptides [7, 15, 16]. Fermented barley and soybean mixtures are also promising anti-photoaging agents [9]. In this study, we discovered the potential peptides derived from tempeh, traditional fermented soybean cake from Indonesia, as anti-photoaging and cosmeceuticals agents. The yields of peptide extracts were 7% w/w for soybean tempeh and 8.3% w/w for black soybean tempeh.

To qualify as a cosmeceutical agent, a peptide should possess one of the following four activities: antioxidant, tyrosinase inhibitor, MMP inhibitor, and anti-inflammatory [17]. Since photoaging activates ROS, the antioxidant activities of a peptide will diminish the ROS to act as anti-aging and increase photo-protective effects [18]. Similarly, photoaging damages the skin by increase MMP activation to promote collagen degradation [19]. Thus, photoaging skin develops wrinkly due to loss of collagen. Therefore, MMP inhibitor activity of peptide could represent anti-wrinkle effect which inhibit collagen degradation [20, 21].

Nonetheless, tyrosinase inhibitor activity frequently associated with whitening effect and anti-inflammatory also corresponded with skin soothing effect [22, 23]. These activities are driven by bioactive peptides consisting specific amino acid sequences derived from specific enzymes [17]. Moreover, the collagen pentapeptide (Lys-Thr-Thr-Lys-Ser, KTKKS), which was reported to promote the production of extracellular matrix and f type I collagen, could permeate the skin effectively, hence it is potential to be used as a cosmeceutical agent [24].

The amino acid compositions in peptide extracts of soybean and black soybean tempeh were quantified based on multiple amino acid standards. More than 20% of the amino acids identified in both peptide extracts were L-glutamic acid. In soybean tempeh peptide extract, the second highest amino acid content was shown by L-aspartic acid (Table 3). Meanwhile, the second major amino acid in black soybean tempeh extract was L-alanine (Table 4). Other amino acid compositions in both tempeh extracts were similar, in which L-histidine was found in least amount.

Amino acid composition predisposes anti-photoaging effect of the peptides. Previous study reported that several amino acid mixtures played an important role in improving skin collagen synthesis rates in UV-irradiated mice [25]. Branched-chain amino acids (BCAA), which consist of leucine, isoleucine, and valine combined with several amino acids, increased collagen synthesis rates significantly after 30 minutes of oral administration. Our black soybean tempeh peptides contained 18.79% BCAA, while soybean tempeh peptides contained 15.04%. In addition, glutamic acid, the main amino acid in black soybean (21.18%) and soybean (23.31%) tempeh peptides was associated with the stimulation of protein synthesis in vitro and in vivo [26].

| Table 3 Amino acid profile of soybean tempeh peptides |
|-----------------------------------------------|
| **Amino acid standards** | **Concentrations (g/kg tempeh powder)** |
| L-Cystine | 1.345 |
| L-Methionine | 5.934 |
| L-Histidine | 12.509 |
| L-Tyrosine | 13.798 |
| L-Serine | 16.614 |
| L-Threonine | 17.798 |
| L-Isoleucin | 18.265 |
| Glycine | 19.431 |
| L-Phenylalanine | 21.874 |
| L-Valine | 22.852 |
| L-Proline | 25.544 |
| L-Alanine | 26.319 |
| L-Leucine | 26.335 |
| L-Arginine | 28.831 |
| L-Lysin HCl | 34.438 |
| L-Aspartic Acid | 51.843 |
| L-Glutamic Acid | 104.515 |

| Table 4 Amino acid profile of black soybean tempeh peptides |
|-----------------------------------------------|
| **Amino acid standards** | **Concentrations (g/kg tempeh powder)** |
| L-Cystine | 0.920 |
| L-Methionine | 5.901 |
| L-Histidine | 8.793 |
| L-Tyrosine | 10.903 |
| L-Serine | 12.864 |
| L-Arginine | 13.895 |
| L-Threonine | 16.093 |
| L-Isoleucin | 17.453 |
| L-Lysin HCl | 17.493 |
| Glycine | 19.205 |
| L-Proline | 19.874 |
| L-Phenylalanine | 20.326 |
| L-Leucine | 23.388 |
| L-Valine | 29.958 |
| L-Aspartic Acid | 33.738 |
| L-Alanine | 46.178 |
| L-Glutamic Acid | 79.838 |

Glutamic acid is an amino acid precursor in proline and hydroxyproline collagen formation. It was reported that in collagen biosynthesis, proline was actively derived from glutamic acid in male guinea pig skin [26]. In addition, the total L-amino acids detected were 448.22 g/kg and 376.82 g/kg dried weight in soybean and black soybean tempeh, respectively. The peptides composed of L-amino acids were reported to protect skin from photoaging and enhance the collagen formation, thus reduced the wrinkles while applied as topical supplementation [27].

3.2 Effect of Tempeh Peptide Extracts on Skin Histology Alteration in UVB-treated Mice

In histological staining, normal skin was characterized by organized structure of epidermis and dermis (Figure 1A). Moreover, normal skin would show active growth of hair follicles and neurons (Figure 1A), while in photoaging skin hair follicle and sub dermis necrosis
would be observed (Figure 1B). The disruption of skin structure involved collagen fiber damages due to UVB radiation [28]. Additionally, photoaging skin is characterized by increase of elastosis and fragmentation of collagen beneath the dermal-epidermal junction [29]. Commercial cosmeceuticals would protect the skin from radiation as characterized by active growth of hair follicles, active neurons, and reduction of dermal-epidermal junction (Figure 1C). In PP100 group, UVB radiation did not affect the formation of hair follicles and repressed the formation of dermal-epidermal junction (Figure 1D). The epidermis thickened was diminished and the hair follicles were reactivated in accordance with dermis layer attenuation in PP300 group (Figure 1E). Similarly, reduced epidermal thickness was reported after the supplementation of soybean and Haematococcus extracts combination against photoaging in vivo [30].

Supplementation of black soybean tempeh peptides in varying doses resulted in histological difference according to nominal scoring. HP100 supplementation could not inhibit the increase of the dermal-epidermal junction and hair follicle degeneration (Figure 1F). Furthermore, the adipose tissue of the skin was thickened in this group, suggesting further photoaging alteration in skin structure [31]. In contrast, HP300 prevented photoaging, as shown by reduction of epidermis-dermis junction thickening, organized subdermis structure and hair follicle growth (Figure 1G).

3.3 Effect of Tempeh Peptide Extracts on Down-regulating Matrix Metalloproteinase-3 and Inflammatory Genes in UVB-induced Mice

Since the histological staining showed significant changes in epidermis formation, we hypothesized that anti-photoaging agents may work through the enzyme inhibitor mechanism of collagenases. These mechanisms can be detected using several biomarkers, such as genes that encode for MMP-1 that initiates the degradation of type I and III collagen, MMP-3 that degrades type IV collagen, and MMP-9 that further decomposes collagen fragments [32]. Our results showed the UVB-treated mice supplemented with both soybean and black soybean tempeh peptide extractsshad down regulation of MMP-3 gene significantly (Figure 2A). In line with our histological examination, the inhibition level of MMP-3 expression by tempeh peptides was higher than UVB-treated PC group by 3.49 fold to PP100, 3.12 fold to PP300, and 4.45 fold to HP300 respectively. Our further examination to MMP-1 and MMP-9 genes showed no significant difference for these genes expression after tempeh peptide supplementation compared to the UVB-treated control (Figures 2B and 2C). This phenomenon may correlated with another activation pathway from MMP promoters directly that allows degradation of type I and III collagens [33]. Repression of MMPs inhibitory activity was also recently reported in UV-treated human Hs68 epidermal fibroblast cells supplemented with myristoyl tetrapeptide Gly-Leu-Phe-Trp (mGLFW) [34].

In addition, the measurement of inflammation-related genes, COX-2 and iNOS indicated that the COX-2 and iNOS biomarker trends were in line with the histological examination although no significant differences were obtained (Figures 2D and 2E). The expression of inflammation-related genes, such as interleukin-1α (IL-1α), IL6, IL-8, IL-10, tumor-necrosis factor α, and proliferator-activated receptor gamma were reported to be abrogated after supplementation of photoaging candidates as one of the hallmark on skin protection [34, 35].

Exposure of UVB radiation led to the upregulation of MMP-encoding genes. It was reported that peptide supplementation of gelatin hydrolysate from pacific cod skin could reduce photoaging effects by inhibiting the expression of MMP-encoding genes and their corresponding proteins [28]. This mechanism is related to UV-irradiation-dependent up-regulated phosphorylation in mitogen-activated protein kinase (MAPK) signaling pathway. Moreover, MMP-9 was secreted by inflammatory cells and activated tumor-associated macrophages, which induced the expression of COX-2 [33]. Repression of COX-2 and iNOS expressions showed the anti-inflammatory properties of tempeh peptide extracts. Another study also demonstrated the increase of p38 kinase, extracellular signal-regulated kinase, c-Jun amino-terminal kinase, and c-Jun phosphorylation in UVB-induced mice supplemented with fermented soybean [36].

Tempeh may be a profitable source of peptides with cosmeceutical properties, as it is a common food product which produces at relatively low cost. Although soybean is the most common ingredient in tempeh making, almost any kind of beans can be fermented using the tempeh starter mold Rhizopus microspores [37]. However, more studies should be conducted to evaluate the cosmeceutical potential of tempeh made with various types of ingredients. Our recent study showed that black soybean tempeh was high in antioxidant activity due to the anthocyanin content in black soybeanhulls [38]. Antioxidants play a role in scavenging ROS in the event of skin exposure to UVB radiation, which consequently prevents collagen degradation [29]. Therefore, such activity will lead to the reduction of skin wrinkling and aging due to UVB radiation.
Figure 1 Representative histological changes in mice dorsal skin following photoaging treatment for 4 weeks. The treatment groups were (A) non-UVB-treated mice (NC), (B) positive control UVB-treated mice (PC), (C) UVB-treated mice supplemented by 2% w/v commercial cosmeceuticals (SP), (D) UVB-treated mice supplemented with 100 mg/kg body weight (bw) soybean tempeh peptide (PP100), (E) 300 mg/kg bw soybean tempeh peptide (PP300), (F) 100 mg/kg bw black soybean tempeh peptide (HP100), and (G) 300 mg/kg bw black soybean tempeh peptide (HP300).
Figure 2 Effect of tempeh peptide extracts on MMP-3 (A), MMP-1 (B), MMP-9 (C), COX-2 (D), and iNOS (E) mRNA expression in mouse skin. The treatment groups were UVB-treated mice supplemented by 2% w/v commercial cosmeceuticals (SP), positive control UVB-treated mice (PC), UVB-treated mice supplemented with 100 mg/kg bw soybean tempeh peptide (PP100), 300 mg/kg bw soybean tempeh peptide (PP300), 100 mg/kg bw black soybean tempeh peptide (HP100), and 300 mg/kg bw black soybean tempeh peptide (HP300). Data were shown as mean ± SD from duplicate experiments. Asterisks (*) indicate significant differences (P ≤ 0.05) compared to PC.

4.0 CONCLUSION

The present study demonstrated the effectiveness of soybean and black soybean tempeh peptide extracts in reducing photoaging effects in mice, as characterized by the significant down-regulation of MMP-3 and inflammation biomarkers-encoding genes. Here, we confirmed the potential use of tempeh peptide extracts as anti-photoaging cosmeceuticals. Further study is needed to reveal the cosmeceutical mechanisms of soybean and black soybean tempeh peptides, presumably via the MAPK pathway.
pathway, and to formulate products for commercialization purposes.

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