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Protection effect of donkey hide gelatin hydrolysates on UVB-induced photoaging of human skin fibroblasts

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ABSTRACT

UVB irradiation is a potent photoaging factor and leads to the formation of skin wrinkles. The objective of this study was to investigate the mechanisms of UVB photoaging inhibition of low molecular weight peptides derived from donkey hide gelatin hydrolysates (LDGH) on human skin fibroblasts. The donkey hide was hydrolyzed using proteases, and the LDGH exhibited higher antioxidant activity than the original hydrolysate and inhibited collagenase and elastase activities. We also found that LDGH increases viability of Hs68 cells after UVB irradiation at 100 mJ/cm^2. Moreover, LDGH prevented the decrease in procollagen type I levels caused by exposure to UVB irradiation in Hs68 cells and reduced the up-regulated phosphorylation of p38, ERK, and JNK in the mitogen-activated protein kinase (MAPK) signaling pathway. These findings suggest that LDGH increases synthesis of procollagen type I by decreasing the phosphorylation of MAPK, therefore, LDGH may be useful as an effective anti-photoaging agent.

1. Introduction

Skin aging is a complex process that can be divided into intrinsic and extrinsic aging. Intrinsic aging is caused by an innate process, which is highly associated with genetic factors. Extrinsic aging, in contrast, is environmentally derived from external factors such as short wavelength UV light (UVB) and characterized by wrinkling and degradation of skin elasticity [1]. Exposure to UVB irradiation causes skin damage by inducing oxidative stress, which leads to DNA mutations in collagenous tissues that produce the extracellular matrix (ECM) with collagen and elastin [2]. UV-induced skin damage leads to activation of the mitogen-activated protein kinase (MAPK) pathway, which comprises c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase. These MAP kinases activated by UV irradiation increase the activation and expression of matrix metalloproteinases, which lead to degradation of type I collagen in human skin fibroblasts [2]. Thus, inhibition of the MAPK pathway can prevent UVB-induced photoaging. Furthermore, preparation of natural products containing compounds with anti-aging effects, such as antioxidant activity, inhibition of the ECM-degrading enzyme (collagenase and elastase), MAPK, and up-regulation of procollagen type I synthesis, may be beneficial against photoaging.

In recent years, diseases induced by UV radiation that cause skin damage became a global concern. In Korea, in particular, it has been reported that the number of people aged 65 or older is continuously increasing. Younger and older people, therefore, would be interested in preventing skin aging, thus increasing the market for anti-photoaging products. Recently, many studies have reported that hydrolysates from bioresources can reduce the risk of skin disease [3,4]. Interestingly, some reports have focused on the beneficial effects of low molecular weight peptides in hydrolysates owing to the anti-photoaging and antioxidant effects in the skin [5,6]. Since the absorption capacity of high molecular weight compound derived from animal collagen may have limitation for real-life application to adapt biological activity, the low molecular hydrolysate or peptides could be more efficient. Nimalaratne et al. [7] reported that low molecular weight peptides are more biologically active compared to its original polypeptide or protein. Furthermore, it is have higher chances to be absorbed to the intestinal membranes by resistance to the gastrointestinal digestion. Similarly, Zhuang et al. [8] reported that oral intake collagen hydrolysate (less than 5000 Da) showed higher effect on restoration of antioxidant enzyme activity and protection of collagen synthesis in mice skin from the UV radiation damages than oral intake of original collagen. In addition, Samaranayaka and Li-Chen [9] reported that antioxidant compounds from food sources have been used to prevent aging and UV-induced skin damage as cosmeceuticals. Many antioxidative peptides have been studied from various food proteins likely egg yolk [10] and royal jelly [11]. Especially, donkey hide has been used in the production of gelatin, a valued traditional medicine and food for skin health in China [12] and Korea. In addition, several bioactivities were identified in

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donkey hide extracts, including antioxidant, neuroprotective, and anti-diabetic effects [13,14]. However, there is still a lack of information on the anti-photoaging activities of enzymatic hydrolysates from donkey hide.

The present study was undertaken to evaluate the antioxidant and the collagenase and elastase inhibitory effects of low molecular weight peptides derived from donkey hide gelatin, and to determine whether these peptides can attenuate UVB-induced MAPK activation and down-regulation of procollagen type I expression in human skin fibroblasts.

2. Materials and methods

2.1. Materials

Donkey hide was obtained from a local donkey farm in Icheon, Korea. Foodpro alkaline protease (Danisco Ltd., Co., Copenhagen, Denmark), pancreatin (Bision Co., Gyunggi, Korea), and protease P (Amano Enzyme Inc., Nagoya, Japan) were used. (±)-6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (97%, Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), fluorescein sodium salt, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,4,6-tripyridyl-s-triazine (TPTZ), collagenase from *Clostridium histolyticum,* 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg, porcine pancreatic elastase, and N-succinyl-$(Ala)_3$-*p*-nitroanilide were purchased from Sigma Aldrich (St. Louis, MO, USA). Trypsin-EDTA, 1% penicillin/streptomycin was purchased from Gibco (Carlsbad, CA, USA) and fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Dulbecco's phosphate buffered saline, Tween 20, acrylamide, Tris-buffered saline (TBS), and sodium dodecyl sulfate (SDS) were purchased from GenDEPOT (Barker, TX, USA). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (DPBS) were purchased from Welgene (Daegu, Korea). All other chemicals and reagents used in this study were analytical grade.

2.2. Preparation of donkey gelatin and low molecular weight donkey gelatin hydrolysates

Donkey hide was washed with water and preheated in boiling water (100 °C, 20 min) to eliminate impurities. After removing the fat layer, donkey hide was chopped into small pieces (1 × 0.5 × 0.5 cm) and then the extraction of gelatin from donkey hide (Fig. 1) was carried out using an autoclave at high temperature and pressure (121 °C, 1.5 kgf/cm^2). The first extraction was carried out by adding 200 mL of distilled water (DW) to 100 g of donkey hide and soaked for 1 h and then filtering using a mesh sieve with less than 150 μm pore size. The second extraction was carried out by adding 100 mL of DW to the sieved donkey hide for 30 min and then filtering again using a similar sieve. The extracts were mixed and enzymatic hydrolysis of gelatin extracts was then performed using Foodpro alkaline protease (F), pancreatin (P), protease P (PP), or a mixture of proteases (Foodpro alkaline protease, pancreatin, and protease P (F/P/PP)) for 3 h (F3, P3, PP3, F/P/PP3) or 6 h (F6, P6, PP6, F/P/PP6) at 45 °C, pH 7.0. Each enzyme was added to the gelatin extracts at an enzyme/substrate ratio (E/S) of 0.3:100. The donkey gelatin hydrolysate (DGH) was then heated at 90 °C for 10 min to inactivate the enzymes. To separate low molecular weight peptides from DGH (LDGH, less than 3 kDa), each DGH was passed through a 3 kDa molecular weight cut-off membrane (Ultracel–3 kDa, Millipore, Ireland). DGHs and the corresponding LDGHs were lyophilized and then stored at −20 °C until use.

2.3. Degree of hydrolysis

Degree of hydrolysis (DH) was determined following the method of Hoyle and Merritt [15] with slight modifications. Protein hydrolysate solution (0.5 mL) was mixed with an equal volume of 20% trichloroacetic acid (TCA). The mixtures were incubated for 30 min at 25 °C and then centrifuged at 3000*g* for 15 min. The soluble protein in the 10% TCA supernatant and total protein contents were determined using the bicinchoninic acid (BCA) protein assay kit (Sigma Chemical, USA). Bovine serum albumin (BSA) was used as a standard. DH was calculated as follows:

DH (%) = [Soluble protein content in 10% TCA/Total protein content in sample] × 100

2.4. Brix and yield

Brix of samples was determined using a refractometer (Master-α, ATAGO, Japan). Yield of samples was determined following the method of Gudmundsson and Hafsteinsson [16]. Yield was calculated as follows:

Yield (%) = [Dry weight of hydrolysate/Wet weight of raw donkey hide] × 100

2.5. Antioxidant activities

2.5.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined following the method of Blois [17] with slight modifications. Reaction mixture containing 100 μL of methanolic solution with DPPH radicals (0.2 mM) and 100 μL of sample was placed in a 96-well microplate. The mixture was shaken and kept in the dark at 25 °C for 30 min, and then absorbance was read at 517 nm using a UV spectrophotometer (Spectramax M2e, Molecular Devices, USA). A standard curve was established using Trolox solution (0, 10, 30, 50, 70, 100, 110, and 120 μM) and the DPPH values were calculated as μM Trolox equivalents per g of dry matter.

2.5.2. ABTS radical scavenging activity

ABTS radical scavenging activity was determined according to Re et al. [18]. Briefly, ABTS radical was produced by reacting a 14 mM ABTS solution with an equal volume of 4.9 mM potassium persulfate in the dark at 25 °C for more than 14 h before use. Next, the ABTS radical solution was diluted in DW and equilibrated at 30 °C to obtain an absorbance of 0.70 ± 0.02 at 735 nm. Sample (50 μL) or Trolox standard solutions (0, 20, 50, 70, 100, 200, 300, and 400 μM in 75 mM PBS) were added to 950 μL of ABTS radical solution and incubated at 30 °C for 30 min in the dark. The absorbance was measured using a UV-spectrophotometer at 735 nm at 30 °C. ABTS radical scavenging activity was calculated using the Trolox standard curve as μM Trolox equivalents per g of dry matter.

2.5.3. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was determined according to the method described by Gillespie et al. [19] with slight modifications. Sample, standards, and other reagents were prepared in 75 mM PBS (pH 7.0). The reaction mixtures containing 150 μL of fluorescein (80 nM) and 25 μL of sample or standard were placed in 96-well black microplates and preincubated for 15 min at 37 °C. After mixing with 25 μL of AAPH (150 mM), the fluorescence was recorded every min for 60 min at 37 °C with emission and excitation wavelengths of 485 and 520 nm, respectively, using a fluorescence reader (Spectramax M2e, Molecular Devices, USA). A standard curve was established by measuring Trolox solution (0, 5, 10, 30, 40, 50, 70, and 100 μM) as area under the curve. ORAC values were expressed as μM Trolox equivalents per g of dry matter.

2.5.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay performed was a slight modification of that described by Benzie and Strain [20]. Briefly, FRAP reagent was freshly