Natural Product Sciences
20(1) : 33-38 (2014)

Inhibitory Effects of Water-soluble Extracts of Barley, Malt, and Germinated Barley on Melanogenesis in Melan-a Cells

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Abstract – In recent times, the demand for edible medication for the treatment of hyperpigmentation has increased significantly. Therefore, the discovery of a stable, safe and inexpansive antimelanogenic component from natural substances, such as grains, is of particular interest. The levels and activities of some metabolites and/or enzymes can be increased. In the present study, we investigated the antimelanogenic effects of water-soluble extracts from barley (BE), malt (ME) and germinated barley (GBE) in melan-a cells. The inhibitory effects of ME and GBE on melanin production were significantly greater than that of BE. Interestingly, the content of ferulic acid, the proposed active component of barley, was also higher in ME and GBE than in BE by HPLC analysis. Western blot analysis of the expression of melanogenic enzymes in melan-a cells treated with BE, ME or GBE indicated the expression of both tyrosinase and tyrosinase-related protein 2 (TRP-2) significantly decreased after treatment with BE, ME or GBE. These results suggest that besides BE, ME and GBE also inhibit melanin production most likely through suppression of tyrosinase and TRP-2 expression. ME and GBE were more efficacious at inhibiting melanin production than BE was and may also represent potential skin-whitening agents.
Keywords – Barley, Malt, Germination, Melanogenesis, Tyrosinase, Tyrosinase-related protein 2

Introduction

Melanin pigments are produced in melanocytes in the basal layer of the epidermis.[1] Melanocytes insert melanin granules into specialized cellular vesicles called melanosomes. Then, the melanosomes containing melanin pigments are then transferred into the neighboring keratinocytes in the human epidermis. The main role of melanin is to protect the skin from UV-induced DNA damage.[2] However, overproduction of melanin may cause cosmetic problems and hyperpigmentary disorders such as freckles, chloasma, and age spots.[3,4] Freckles, clusters of concentrated melanin found in 34% of the skin area in women and appear from the age of 34-35 years, 50% of women have chloasma by the age of 50 to 55 years. Laser treatments are often used to reduce the appearance of freckles. Laser whitening is a fast and painless procedure with long-lasting results. However, it can further worsen skin conditions, because it has side effects such as redness, itching, and increased sensitivity to sunlight. Therefore, the discovery of a stable, safe and inexpensive anti-melangenic molecule from natural substances such as fruits, grains and vegetables is of particular interest.

Melanogenic enzymes regulate melanin production and skin whitening and are therefore essential for the regulation of skin whitening. Melanogenesis is mainly regulated by tyrosinase, tyrosinase-related protein 1 (TRP-1) and 2 (TRP-2).[5] Tyrosinase is a bifunctional enzyme that plays a significant role in the modulation of melanin production by catalyzing the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to DOPAquinone. TRP-2 catalyzes the rearrangement of DOPAchrome to 5,6-dihydroxyindole 2-carboxylic acid (DHICA)[6] and TRP-1 oxidizes DHICA to indole-5-6-quinone carboxylic acid.[7] In mammals, two types of melanins are formed; eumelanin (brown/black) and pheomelanin (yellow/red). Tyrosinase is required for synthesis of both eumelanin and pheomelanin. TRP-1 and TRP-2 are more crucial for the synthesis of eumelanin than for the synthesis of pheomelanin.[8]

Barley (*Hordeum vulgarea* L.) is one of the world's

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most consumed grains, and has been recommended as a particularly healthy supplement for the prevention of various diseases.[9] Barley contains several phenolic compounds with the most common ones being, catechin, epicatechin, proanthocyanidin, quinone as well as ferulic, vanillic, and *p*-coumaric acids.[10-12] These compounds have anti-hyperlipidemic[13,14] and antioxidative effects,[15] can also prevent cardiovascular disease,[16,17] and reduce inflammation[18] and act as tumor suppressive agents.[19] Although barley has been reported to show various pharmacological activities, to our knowledge, the present study is the first to describe the effect of barley on skin melanogenesis. Malt is processed from barley and is germinated under specific water, temperature, and oxygen conditions. The levels of phenolic compounds, ergosterol, amino acids, hordenine, gramine, and α-amylase may be changed during the malting process of barley. The antioxidative properties of barley and malt are mainly attributable to the presence of phenolic compounds, especially flavonoids and hydroxycinnamic acids.[20,21] Germinated barley is processed from the barley, like malt, but in contrast to malt, it has no roots. Previous study had reported that malt extracts contained not only rootlet oil, proteins, sugars, and phenolic compounds, but also candicine.[22,23] Therefore, barley, malt and germinated barley may have some difference in physiological effects, respectively.

In this study, we investigated and compared the inhibitory effects of water-soluble extracts of barley (BE), malt (ME) and germinated barley (GBE) on melanogenesis, and measured the content of ferulic acid, the proposed active compound of barley in each sample by HPLC analysis. Moreover, melanin contents and cell viability as well as the expression level of the melanogenic enzymes in melan-a cells were evaluated after treatment with BE, ME or GBE.

Experimental

Materials – RPMI1640 media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-streptomycin (PS) was purchased from Invitrogen (Grand Island, NY, USA). All other chemicals including ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were of the highest purity grade. Primary and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA), respectively.

Preparation of extracts – Barley, malt and germinated barley were provided by Yeonggwang Agricultural Technology Center (Jeollanam-do, Korea). Each (500 g) was extracted three times with distilled water for 1 hr under ultrasonication. The extracts were filtered and lyophilized. The yield of extract for barley was 8.06%, while that for malt and germinated barley was 6.77%, and 7.13%, respectively.

Determination of ferulic acid content in BE, ME and GBE by HPLC analysis – A standard stock solution was prepared by dissolving 1 mg of ferulic acid in 1 ml methanol and stored at –20 ºC. Water-soluble extracts of barley (BE), malt (ME) and germinated barley (GBE) (500 mg) were dissolved in 5 ml methanol, then incubated in an ultrasonic bath for 60 min, filtered, and then injected in the HPLC system. HPLC analysis was conducted using a Waters system (Waters Corp., Milford, MA, USA) consisting of separation module (e2695) with an integrated column heater, an autosampler and a photodiode array detector (2998). UV absorbance was monitored between 200 and 400 nm. Quantification was carried out by integration of the peak areas at 325 nm. Injection volume was 10 μl. A column (J'sphere ODS-H80, 250 × 4.6 mm; particle size, 4 μm; YMC Co. Ltd., Japan) was installed in a column oven and maintained at 40 ºC. The mobile phase consisted of water containing 1% acetic acid (solvent A) and a gradient of methanol (solvent B); 0 min, with 10% solvent B; 15 min, with, 35% B; 30 min, with 35% B; 70 min, with 90% B; 75 min, with 90% B; 80.0 min, with 10% B. The flow rate was 1.0 ml/min. The re-equilibration time between runs was 20 min.

Cell culture – Murine melanocyte melan-a cells were kindly supplied by Dr. Byeong Gon Lee at the Skin Research Institute, Amore-Pacific Co. (Yongin, Korea). Melan-a cells were maintained in RPMI1640 mediun supplemented with 10% FBS, 1% PS, and 200 nM 12-*O*-tetradecanoyl phorbol 13-acetate. The cells were incubated at 37 ºC in a humidified incubator with 5% CO_2.

Determination of melanin contents and cell viability – The melan-a cells were seeded at 1×10^5 cells/well in 24-well plates and incubated at 37 ºC for 24 hrs. The medium was changed daily, and cell were treated for 72 h with 10, 100 and 250 μg/ml (final concentration) of test samples. The content of melanin pigments was measured by a slight modification of the method described by Hosoi *et al*.[24] The wells were washed with PBS after the media was removed from each well. Then, 500 μl of 1 N NaOH was added to the cells, and the plates were gently agitated to dissolve the melanin. The absorbance was measured at 405 nm by using a microplate reader. Cell viability was assessed using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-

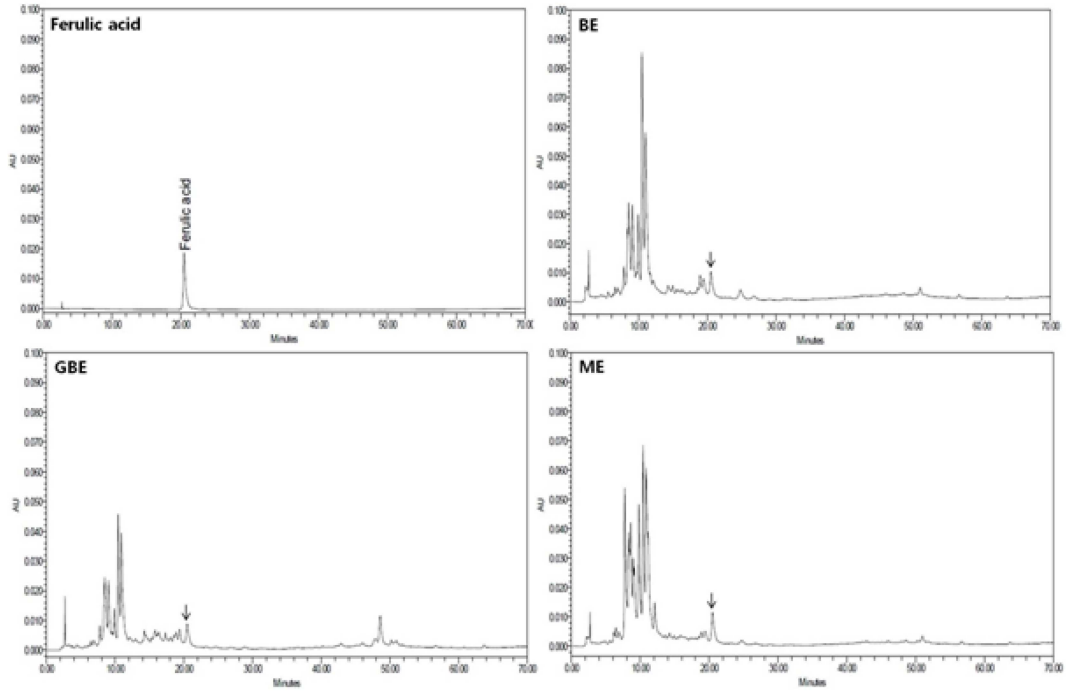

Fig. 1. HPLC chromatograms of ferulic acid, BE, ME and GBE.

tetrazolium bromide (MTT) assay.

Western immunoblotting analysis – Melan-a cells were treated with BE, ME and GBE for 72 h. The cells were then harvested and protein extract was performed using a triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml PMSF, and 1 μg/ml aprotinin). The protein concentraction was measured using a protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of proteins were separated on a 8% SDS-polyacrylamide gel and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 5% w/v skim milk and incubated overnight with the corresponding primary antibodies at 4 ºC. After several washes with the blocking buffer, the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Immunoreactive proteins were detected using ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical Analysis – Data were expressed as means ± S.D. from at least three independent experiments. The student's *t*-test was used for statistical analysis, and only *p*-values of < 0.05 were reported as significant. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ indicate statistically significant differences from the control group.

Table 1. Contents of feruric acid in BE, ME and GBE

Samples	r^2	Ferulic acid (μg/mg)
BE		0.0356 ± 0.0003
ME	0.9989	0.0426 ± 0.0003
GBE		0.0533 ± 0.0003

Results and Discussion

Using HPLC, it was found that BE, ME and GBE contained ferulic acid (Fig. 1). The retention time for ferulic acid was 20.437 min. The amount of ferulic acid contained in BE, ME and GBE was determined from three independent samples. The correlation coefficient (r^2) was 0.9989. The concentrations of ferulic acid in BE, ME and GBE were 0.0356 ± 0.0003 μg/mg, 0.0426 ± 0.0003 μg/mg, and 0.0533 ± 0.0003 μg/mg, respectively (Table 1).

The ability of BE, ME and GBE to inhibit melanin production was assessed using a cell-based assay. As shown in Fig. 2A, 100 and 250 μg/ml of BE, ME and GBE significantly decreased the melanin production in melan-a cells. Treatment with 100 μg/ml BE and ME inhibited the cellular melanin content by approximately 41.2% and 40.2%, respectively, while that with 250 μg/ml of BE and ME inhibited the cellular melanin content by 44.9% and 53.6%, respectively. GBE was more potent than BE or ME. The GBE reduced melanin production by

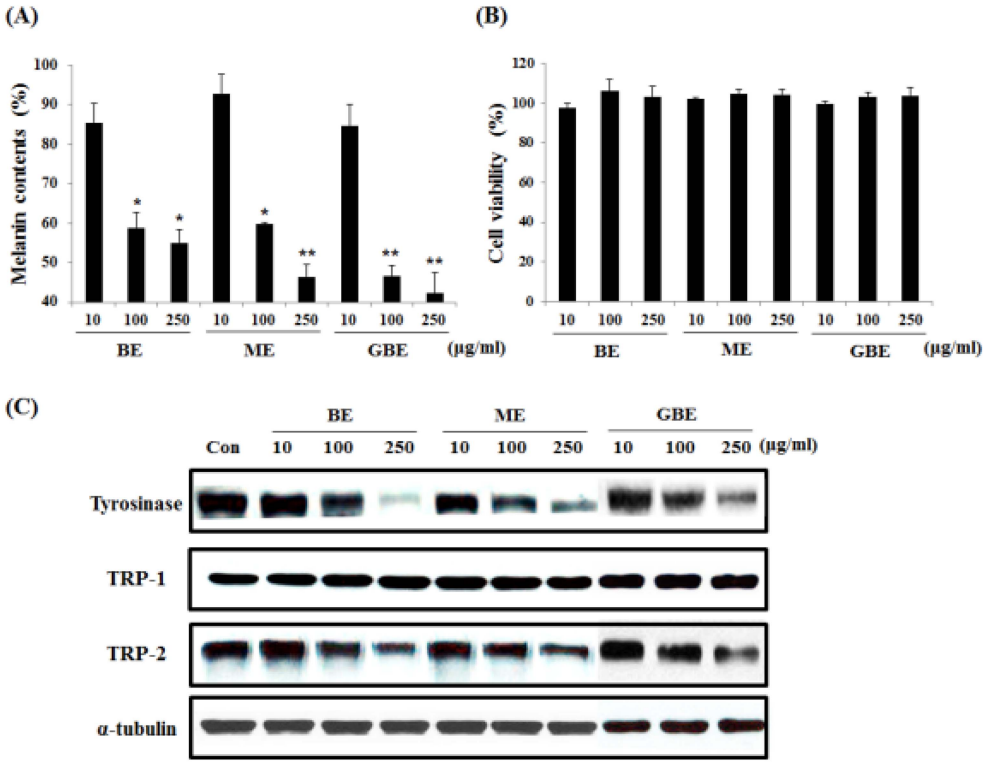

Fig. 2. Effects of BE, ME and GBE on melanin production in melan-a cells. To evaluate (A) melanin production, (B) cell viability and (C) melanogenic enzyme expression, melan-a cells were treated with 10, 100, 250 μg/ml of BE, ME and GBE for 72 h. The results are averages of three independent experiments, and the data are expressed as means ± SD (* : p-value < 0.05 and ** : p-value < 0.01).

53.3 and 57.6% after treatment with 100 and 250 μg/ml, respectively, compared to the control. In our previous study, 272 μg/ml of arbutin inhibited melanin content by approximately 50%.[25] The viability of melan-a cells after treatment with BE, ME and GBE was also tested using the MTT assay. Compared to the untreated control, as shown Fig. 2B, none of the extracts any significant cellular toxicity at any concentration tested.

To examine the effect of barley extracts on protein expression of melanogenic enzymes, we treated samples in melan-a cells and performed Western blot analysis. As shown in Fig. 2C, treatment with 10, 100 or 250 μg/ml of BE, ME and GBE for 72 h down-regulated the expression levels of tyrosinase and TRP-2 in a dose-dependent manner. However, they had no effect on TRP-1 protein expression.

It has been previously reported that barley has various pharmacological properties such as anti-hyperlipidemic effects,[14,26] antioxidative properties,[7] and anti-inflammatory and tumor-suppressive effects. However, to our knowledge, no report regarding the effect of barley on melanin biosynthesis has been published. In this study, we aimed to investigate the anti-melanogenic effects of BE, ME and GBE on murine melanocyte melan-a cells as well as to elucidate the underlying mechanisms. The BE, ME and GBE showed antimelanogenic effects without significant cell toxicity. ME and GBE were more effective at inhibiting the melanin production than the BE was. These results suggest that the germination process might increase the antimelanigenic properties of barley, most likely through an increase of ferulic acid content.

Because ferulic acid is a well- known powerful antioxidant, many dermatological studies have already reported its effect. A topical antioxidant complex containing ferulic acid has been report for its potential photoprotetive effects against UVR-induced acute photodamage on human skin.[27] In addition, ferulic acid has been reported to its effects in reducing UVA-mediated metalloproteinase-1 concentraction in HaCaT cells, possibly through restoration of the antioxidant defense system.[28] Oxidation plays a critical role not only in skin photoaging but also in the melanogenic pathway. The production of the melanin precursors, dopaquinone and dopachrome, occurs through an oxidation process mediated by tyrosinase. In recent years, amino acids and Vit E derivatives as well as ferulic acid have been reported to have skin whitening and anti-photoaging. Also, it had reported that ferulic acid showed considerable inhibitory activity against mushroom tyrosinase and reduction of melanin synthesis in B16 melanoma

cells.[29]

Previously, Federico *et al.* reported that free water-soluble phenolic compounds such as lutonarin, *p*-coumaric acid, saponarin, and isoorientin as well as ferulic acid were contained in barley.[11] Barley, malt, and germinated barley exhibit antioxidant properties mainly due to the presence of these phenolic compounds, especially flavonoids and hydroxycinnamic acids.[22]

Candicine, is an alkaloid found in malt rootlet, and is a toxic compound causing a neuromuscular block.[23] In this study, GBE was more potent for depigmentation activity than ME was. Moreover, germinated barley has no rootlets unlike malt. Therefore, GBE may be not only a more effective but also a safer skin whitening agent than ME.

In order to determine the chemicals that represent the difference of anti-melanogenic effets, ferulic acid, one of the most antioxidative chemicals in barley, was determined by HPLC analysis. BE and ME, respectively, contained and 0.0356 ± 0.0003 μg/mg and 0.0426 ± 0.0003 ferulic acid. The concentration of ferulic acid in GBE was slightly higher 0.0533 ± 0003 μg/mg. This seem to indicate that the concentraction of ferulic acid up-regulated during barley germination. Tian *et al*. reported that the total content in phenolic compounds was significantly higher in germinated brown rice than in ungerminated rice, and that ferulic acid was the most abundant phenolic compound in germinated rice.[30] Additional active molecules with anti-melanogenic effects may be present in barley. On the basis of our HPLC analysis, we hypothesize that such molecules would be highly polar compounds, and this needs to be further investigated. Namely, it is possible that there are more active ingredients for antimelanogensis besides ferulic acid in barley. We suggest this because there is not so much amount of ferulic acid exist in barley, ME or GBE (Fig. 1).

To investigate the anti-melanogenic mechanisms of barley, the effects of BE, ME and GBE on the expression of the melanogenic enzymes, tyrosinase, TRP-1 and TRP-2, were examined by western blot analysis. BE, ME and GBE down-regulated the expression of tyrosinase and TRP-2 proteins but not that of TRP-1. Tyrosinase is a key enzyme that catalyzes the rate-limiting conversion steps in melanogenesis. TRP-2 isomerizes DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA)[31] and activates the DHICA-mediated eumelanin production.[32] Therefore, BE, ME and GBE induce a hypopigmentary effect *via* inhibition of tyrosinase and TRP-2 levels. In this study, ME and GBE did not appear more efficacious at inhibiting the expression of tyrosinase and TRP-2 than BE. However, they induced a more significant decrease in melanin production compared to hat induced by BE. Therefore, it is possible that the potent anti-melanogenic effects of ME and GBE resulted from a variety of mechanisms from synergy between multiple compounds produced during germination. We also investigated the effects of BE, ME and GBE on tyrosinase and TRP-2 activities in melan-a cell-originated enzyme activity assays. However, none of the tested samples influenced either enzyme activity (data not shown). Therefore, we hypothesized that the antimelanogenic effects of BE, ME and GBE were not caused by an inhibition of the activity of tyrosinase and TRP-2, but rather by reduction in their expression level.

In conclusion, we investigated the effects of BE, ME and GBE on melanin biosynthesis in melan-a cells. BE, ME and GBE significantly reduced melanin production and also inhibited tyrosinase and TRP-2 expression. Our results suggest that BE, ME and GBE suppress melanogenesis *via* inhibition of the levels of tyrosinase and TRP-2 protein expression. The current results also indicate that a new non-toxic edible germinated barley may be useful sources of natural skin-whitening agents. Future work is needed to investigate whether ferulic acid is responsible for the effects observed, and to identify other potential polar active substances present in malt- and germinated barley extracts. In addition, it is necessary to investigate the skin whitening efficacy due to the dietary intake of germinated barley.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NO. 2011-0014559). Thanks to Julia Yeiun Kim from Korea International School for partly assisting research.

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Received September 23, 2013
Revised October 21, 2013
Accepted October 27, 2013