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[1]Department of Industrial Plant Science and Technology, College of Agricultural, Life and Environmental Sciences, Chungbuk National University, Republic of Korea
[2]College of Agriculture & Life Sciences, SARI, Jeju National University, Republic of Korea
[3]Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Republic of Korea

Influence of ripening stages on phytochemical composition and bioavailability of ginseng berry (*Panax ginseng* C.A. Meyer)

Sora Jin[1], Seung Hee Eom[1], Ju-Sung Kim[2], Ick-Hyun Jo[3*], Tae Kyung Hyun[1*]

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Summary

The presence of large amounts of bioactive compounds such as saponins and flavonoids in ginseng (*Panax ginseng*) berry suggests its potential as a functional resource for the food and medical industries, despite the fact that been considered a useless by-products of *P. ginseng*. In this study, we examined the variations in the antioxidant and anti-melanogenic potential of ginseng berry during the ripening process. We found that fully ripe berry extracts (Go-S3) contained the highest level of antioxidant and anti-melanogenic activities. Phytochemical screening suggested that alterations in polyphenol contents correlated with the variation in bioactive principles of ginseng berry during the ripening process. Furthermore, results obtained by quantitative real-time PCR, western blot, tyrosinase inhibition assay and molecular docking analysis suggested that Go-S3 probably inhibits tyrosinase activity by interacting with copper-coordinating histidines and second shell residues of tyrosinase, resulting in the reduction of melanin production in α-MSH-stimulated B16F10 cells. Taken together, these finding suggest the potential of ginseng berry as a resource for functional applications in the cosmetic industries and demonstrate that fruit ripening stages have profound effects on the pharmaceutical value of ginseng berry.

Keywords: *Panax ginseng*, antioxidant activity, melanogenesis, anthocyanin, molecular docking.

Introduction

Melanin is a pigment found in the skin, hair and eyes; it plays a role of protecting the skin from ultraviolet light damage. However, overproduction of melanin leads to various pigmentation disorders, including melasma, solar lentigo and hyperpigmentation, and to autoimmune disorders such as vitiligo (Briganti et al., 2003; Niu and Aisa, 2017). Melanogenesis, the process of melanin synthesis in the melanocytes, begins with L-tyrosine and proceeds through a series of enzymatic and chemical reactions initiated by tyrosinase, an oxidase that is the rate-limiting enzyme for the two initial enzymatic steps in the conversion of tyrosine to melanin (Pillaiyar et al., 2017). In addition, tyrosinase related proteins 1 and 2 (TRP-1 and TRP-2) also contribute to the production of melanin. Furthermore, accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) promotes melanogenesis by increasing the transcription, translation and activities of tyrosinase in melanocytes and/or melanoma cells (Liu-Smith and Meyskens, 2016). This suggests a connection between oxidative stress and various pigmentation disorders and suggests that removing ROS or the inhibition of oxidative reactions may have promising depigmenting properties. In fact, anti-melanogenic effects of plant-derived antioxidants have been intensively explored (Liu-Smith and Meyskens, 2016; Kanlayavattanakul and Lourith, 2018).

The inedible portion of vegetables and some fruits range from 25 to 30% of total production (Ajila et al., 2010). Despite the fact that by-products or residual biomasses of medicinal plants should be used with care because of undesirable side-effects, by-products of several plants, may be inexpensive and alternative source of antioxidants. Therefore, utilization of plant by-products will significantly contribute to economic savings.

Ginseng (*Panax ginseng* C.A. Meyer) is a perennial herb, belonging to the Araliaceae family. Ginseng root is the most popular medicinal herb in the world, whereas the berry has been considered a useless by-product (Hyun and Jang, 2017). Since it has been reported that the berries contain more ginsenoside Re than the roots (Attele et al., 2002), recent interest on the part of the food and medical industries has focused on the potential of ginseng berry as a beneficial biomaterial. Therefore, in this study, we evaluated the pharmaceutical value of ginseng berry by analyzing its antioxidant activity and anti-melanogenic effects and analyzed the variation in bioactivities of ginseng berry during the ripening process. Furthermore, to investigate the underlying molecular mechanism by which ginseng berry suppresses melanogenesis, we analyzed the transcription, translation and activity of tyrosinase as well as *in silico* molecular docking and preformed high performance liquid chromatography (HPLC) analysis to determine the existence of various active compounds including ginsenosides and anthocyanins.

Material and methods

Plant materials and extraction

P. ginseng cultivar (Gopoong) was grown at the Department of Herbal Crop Research, Rural Development Administration, South Korea. Ginseng berries were collected from 50 individual plants. Berry samples were collected during the three fruit ripening stages: green fruit (unripe stage), red-turning fruit (semi-ripe stage) and dark red fruit (fully-ripe stage) (Fig. 1A). The freeze-dried samples were soaked in 70% ethanol (EtOH) for 24 h, placed in an ultrasonic bath and sonicated at 55 °C. After filtration, the 70% EtOH extracts were evaporated using a rotary vacuum evaporator.

Determination of total phenolic (TPC), flavonoid (TFC), carotenoid (TCC), saponin (TSC) and anthocyanin contents (TAC)

TPC and TFC were determined according to the Folin-Ciocalteu method and colorimetric method, respectively, as described by Hyun et al. (2016). The TPC in the 70% EtOH extracts was expressed in milligram gallic acid equivalent (mg GAE/g extract) using the equation obtained from the standard gallic acid graph. TFC was determined as milligrams of quercetin equivalents (QE) per gram of extract (mg QE/g extract).

* Corresponding author

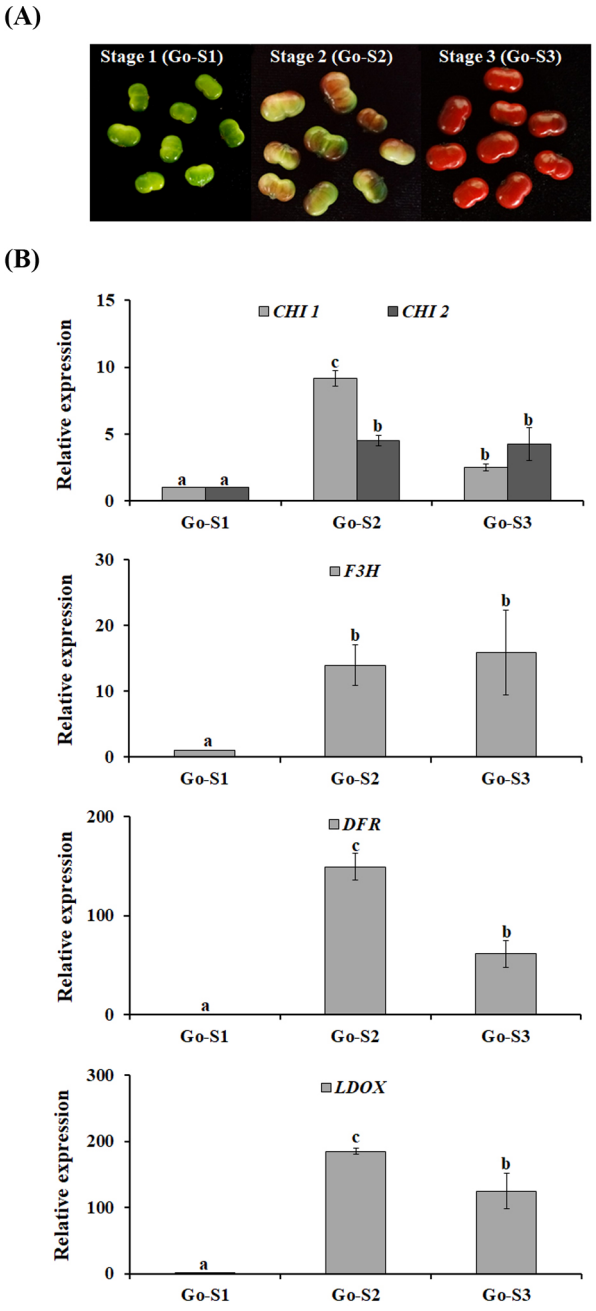

Fig. 1: The expression pattern of anthocyanin biosynthesis genes at various stages of the ripening process. **(A)** *Panax ginseng* berry ripening stages considered in this study. **(B)** The expression levels for each gene were calculated relative to their expression in stage 1. Enzyme names were abbreviated as follows: chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX).

To analyze TCC in 70% ethanol extracts, the absorbance was determined at 490 nm (OD480), 510 nm (OD510) and 750 nm (OD750), respectively, using iMark^{TM} microplate reader (Bio-Rad, Hercules, CA, USA). TCC was calculated using following formula, as described by PARSONS and STRICKLAND (1963):

Total carotenoid (μg/mg of extract) = 7.6 × (OD480 - OD750) - 1.49 × (OD510 - OD750)

For TAC analysis, 10 mg of each extract was dissolved in 1 ml of acidified methanol (1% HCl), and the absorbance was measured at 530 nm (OD530) and 657 nm (OD657) as described by CHOI et al. (2018). TAC was calculated as follows:

Total anthocyanin = (OD530-0.25 × OD675)/ 10 mg of extract

TSC was determined by the colorimetric method (LEE et al., 2018) using diosgenin as a standard. Briefly, 10 mg of each extract was dissolved in 1 ml ethyl acetate, and mixed with 500 μl A reagent (0.5 % (v/v) p-anisaldehyde in ethyl acetate) and 500 μl B reagent (50% (v/v) H_2SO_4 in ethyl acetate). After incubation at 60 °C for 10 min, the absorbance was measured at 430 nm. TSC was calculated as diosgenin equivalent from the equation obtained from the standard diosgenin graph.

Determination of antioxidant activity

To analyze the free radical scavenging activity of each sample, 90 μl of 0.4 mM 1,1-diphenyl-2-picryl-hydrazil (DPPH) was mixed with different concentrations (62.5 to 1000 μg/ml) of each test sample. After incubation for 10 min at room temperature, absorbance was measured at 520 nm using a spectrophotometer. The RC50 (50% reduction of DPPH radicals) was calculated from a graph of radical scavenging activity vs. extract concentration.

The reducing power of each sample was determined according to the method of CHOI et al. (2018). Various concentrations of extracts (100, 200, and 300 μg/ml) were mixed with 0.2 ml of 200 mM sodium phosphate buffer (pH 6.6) and 0.2 ml of 1% potassium ferricyanide. After incubation at 50 °C for 20 min, the mixture was mixed with 1 ml of 10% trichloroacetic acid, and centrifuged at 6,500 rpm for 10 min. Then 500 μl aliquots of the supernatants were mixed with 500 μl of deionized water and 100 μl of 0.1% ferric chloride, and the absorbance was measured at 750 nm. Ascorbic acid was used as a positive control.

The oxygen radical antioxidant capacity (ORAC) assay was conducted according to CHOI et al. (2017). A total of 150 μl of 0.08 μM fluorescein diluted in phosphate buffer (75 mM, pH 7.0) was mixed with 25 μl of phosphate buffer (blank), Trolox standard (6.25-50 μM), or each extract in separate wells of microplate. After incubation at 37 °C for 10 min in dark, 25 μl of fresh 2,2'-azobis(isobutyramidine) dihydrochloride (0.12 g/ml) was added. The fluorescence intensity was monitored using 485 nm excitation and 525 nm emission wavelengths at 1 min intervals for 90 min in a SpectraMax Gemini EM microplate reader (Molecular Devices, CA, USA). The area under the curve was calculated for each sample by integrating the relative fluorescence curve. ORAC values were expressed as μM of Trolox equivalents (μM TE).

Cell viability assay and determination of melanin synthesis inhibitory activity

The B16F10 melanoma cell line was purchased from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin in an incubator containing humidified CO_2 (5%) at 37 °C. Cultured B16F10 cells were plated at a density of 1×10^5 cells/ml in 96-well plates and incubated at 37 °C for 24 h. Then, the cells were treated in each concentration of extracts with or without 50 nM of α-melanocyte-stimulation hormone (α-MSH). After incubation for 48 h, the medium was replaced with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) for 4 h. The formazan crystals were dissolved in DMSO, and the absorbance was measured at 520 nm using an iMARK microplate reader (Bio-RAD).

For the determination of melanin synthesis inhibitory activity, cultured B16F10 cells were plated at a density of 1×10^5 cells/ml in 6-well plates and incubated at 37 °C for 24 h. B16F10 cells were treated with each extract and 50 nM α-MSH and incubated at 37 °C for 48 h. The cells were washed twice with ice-cold PBS and harvested at

4,000 rpm for 10 min. The pellets were solubilized in 1 N NaOH with 10% dimethyl sulfoxide at 65 °C for 1 h. The amount of melanin was determined by a microplate reader at 490 nm. Data are expressed in terms of melanin synthesis inhibitory activity compared to the mock control

Tyrosinase inhibition assay

The tyrosinase inhibitory activity of ginseng fruit extract was measured using Tyrosinase Inhibition Screening Kit (BioVision, CA, USA) according to the manufacturer's instructions. After incubation for 30 min at room temperature, tyrosinase activity was determined by a microplate reader at 510 nm. Ascorbic acid was used as a positive control.

Western blot analysis

After treatment with various concentrations of extract in the presence of 50 nM α-MSH for 48 h, B16F10 cells were analyzed by immunoblotting. The cells were washed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxylcholate, 1 mM EDTA, and 10 mM NaF). The concentration of protein extracts was determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA). Then, equal amount (20 μg) of proteins was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). After blocking with 5% non-fat dried milk, the membranes were hybridized with specific antibodies. The signal was detected and visualized using a chemiluminescence system according to the manufacturer's instructions.

Expression analysis using quantitative Real-Time PCR (qRT-PCR)

B16F10 cells were treated with α-MSH and extracts as described above, and total RNA was extracted using TRIzol reagent (Molecular Research Center, Cincinnati, USA). In addition, total RNA from ginseng berries was isolated using the FavorPrep Plant Total RNA Purification Mini Kit (Favogen, PingTung, Taiwan) according to the manufacturer's instructions. Total RNA content and purity were assessed using a DeNovix DS-11 spectrophotometer (Denovix, UL, USA). Then, 100 to 250 ng of total RNA were reverse-transcribed into cDNA using the ReverTra Ace® qPCR RT Master Mix with qDNA Remover (TOYOBO, Co., Ltd, Osaka, Japan) in accordance with the manufacturer's recommendations. qRT-PCR was performed using the SYBR® Green Real-time PCR Master Mix (TOYOBO, Co., Ltd, Osaka, Japan) in the CFX96™ Real-time system (BIO-RAD) with default parameters. The expression levels of each gene were normalized to actin, and the specific primer pairs used in qRT-PCR are listed in Tab. S1.

HPLC analysis

The composition of anthocyanins and ginsenosides in ginseng berries was determined using Agilent Technologies 1200 series HPLC (Conquer scientific, CA, USA) with COSMOSIL 2.5 C_{18} Cholester (2.0ID × 50 mm, for anthocyanin analysis) or Kinetex 2.6u XB-C18 100A (100 × 4.6 mm, for ginsenoside analysis). For anthocyanin analysis, 10 mg of each extract was dissolved in 1 ml of acidified methanol (1% HCl). The mobile phases consisted of 1% phosphoric acid in water (solvent A) and acetonitrile (solvent B). In the case of ginsenoside analysis, 10 mg of each extract was dissolved in methanol. HPLC analytical conditions are described in Tab. S2. Standard calibration curves of cyanidin-3-glucoside (C3G, Sigma-Aldrich Co., St. Louis, MO, USA), delphinidin-3-glucoside (D3G, CoreSciences, Seoul, Korea), petunidin-3-glucoside (P3G, Sigma-Aldrich Co., St. Louis, MO, USA) and Ginseng Ginsenosides Mix (Sigma-Aldrich Co., St. Louis, MO, USA) were constructed.

Molecular docking

Molecular blind docking and defined docking were performed using tyrosinase (PDB code, 3NQ1), cyanidin-3-glucoside (PubChem CID, 44256715), delphinidin-3-glucoside (PubChem CID, 443650), and petunidin-3-glucoside (PubChem CID, 443651). AutoDock4 with Lamarckian Genetic Algorithm (Morris et al., 2009) was used to calculate the docking results. Molecular docking analysis was performed using the protocol described by Seo and Efferth (2016). For blind docking, grid box was constructed to cover whole residues, and docking parameters were set to 100 runs and 2,500,000 energy evaluations for each cycle. Based on blind docking results, grid maps were created for defined docking. Docking parameters of defined docking were set to 250 runs and 2,500,000 energy evaluations each time. The lowest binding energies and predicted inhibition constants were obtained from the docking log files.

Statistical analysis

All experiments were conducted with three independent replicates, and Duncan's test was used to determine the significance of differences between the groups. Differences at $p < 0.05$ were considered significant.

Results and discussion

Variation in phytochemical contents at the three ripening stages of ginseng berry

Various environmental and genetic factors, including seasonal variations, growth conditions, stages of maturity and cultivars have great influence on alterations in the accumulation and composition of phytochemicals in medicinal plants (Cordenunsi et al., 2002). As a first step, in order to determine the variability in bioactive principles during the fruit ripening process, we compared phytochemical contents, including total phenolic (TPC), flavonoid (TFC), carotenoid (TCC), saponin (TSC) and anthocyanin (TAC) contents in the various maturity stages of ginseng berries. TPC, TFC and TAC generally increased during the progression of ripening, whereas TCC was higher in extracts from unripe samples (Go-S1) than in semi-ripe (Go-S2) or fully ripe (Go-S3) samples (Tab. 1). This suggested that anthocyanins considerably contribute to color differences during the ripening process of ginseng berry. Go-S2 contained the highest amount of saponin compounds (9.79 ± 2.98 μg/mg of extract). The content and composition of ginsenosides, pharmacologically active compounds found exclusively in the genus *Panax* of the family Araliaceae (Pace et al., 2015), exhibit variation among tissues, cultivation ages and environmental conditions (Shan et al., 2014; Zhang et al., 2014). Based on HPLC analysis, we detected five ginsenosides: Rb2, Re, Rf, Rg1 and Rg2 (Tab. 2). The amounts of Re, Rf, Rg1 and Rg2 decreased during the progress of ripening, whereas the highest amount of ginsenoside Rb2 was found in Go-S2. It has been reported that the most abundant ginsenosides in ginseng leaf and root are Re and Rb1, respectively (Kim et al., 2014). In the present study, Rg1 appeared to be most abundant in ginseng berries (Tab. 2). Triterpenoid saponins are constitutively synthesized in the leaves, then transported to the roots via the phloem (Eom et al., 2017). Tissue-specific expression of cytochrome P450s (P450s) and UDP-dependent glycosyltransferases (UGTs) is crucial for structural diversity and functionalization of triterpenoid saponins (Kumar et al., 2012; Wang et al., 2018). This suggests that the differential distribution of ginsenosides among tissues might be due to the tissue-specific expression pattern of genes involved in the modification of triterpenoid saponins, including *P450s* and *UGTs*.

Tab. 1: The effect of various maturity stages of ginseng berry on phytochemical contents.

Sample	Remark	Total flavonoid (mg QE/100 mg)[a]	Total phenol (mg GAE/100 mg)[b]	Total saponin (μg/mg of extract)	Total carotenoid (μg/mg of extract)	Total anthocyanin ((OD530-0.25*OD675)/mg of extract)
Stage 1	Go-S1	0.45±0.01	11.78±3.05	7.04±1.15	0.43±0.09	0.032±0
Stage 2	Go-S2	0.47±0.01	16.23±2.89	9.79±2.98	0.32±0.07	0.055±0.001
Stage 3	Go-S3	0.62±0.01	20.28±2.34	7.05±2.52	0.31±0.01	0.200±0.004

[a] Total flavonoid content analyzed as quercetin equivalent (QE) mg/100 mg of extract; values are the average of triplicate experiments
[b] Total phenol content analyzed as gallic acid equivalent (GAE) mg/100 mg of extract; values are the average of triplicate experiments

Tab. 2: Content of ginsenosides and anthocyanins of ginseng berry in different ripening stage.

	Ginsenosides (μg/g of extract)					Anthocyanins (μg/g of extract)		
	Rb2	Re	Rf	Rg1	Rg2	Delphinidin-3-glucoside (D3G)	Cyanidin-3-glucoside (C3G)	Petunidin-3-glucoside (Pt3G)
Go-S1	93.36±6.89	110.68±6.09	198.55±7.11	213.67±6.56	180.55±7.95	1.1±0.1	0.5±0.2	-
Go-S2	99.31±7.43	103.52±11	130.33±9.46	150.02±9.08	121.73±7.18	239.6±1.7	233.4±2.0	1.1±0.0
Go-S3	83.83±5.1	103.58±10.4	126.24±10.3	139.55±5.4	111.64±7.2	541.8±5.4	462.8±11.7	1.1±0.0

Anthocyanin accumulation occurring during fruit ripening is an important physiological process for attracting fruit-eating animals and hence dispersal of seeds (Zifkin et al., 2012). In higher plants, the most common types of anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin; of these, cyanidin is a major anthocyanidin of berries and red vegetables (Khoo et al., 2017). In the case of ginseng berry, delphinidin-3-glucoside (541.8 ± 5.4 μg/g of extract) was the most abundant anthocyanin followed by cyanidin-3-glucoside (462.8 ± 11.7 μg/g of extract) (Tab. 2). Briefly, biosynthesis of anthocyanin originates from the general phenylpropanoid pathway, followed by the early flavonoid pathway and the anthocyanin-specific pathway (Choi et al., 2018). During fruit ripening, the accumulation of anthocyanin is positively associated with the transcription of genes involved in the early flavonoid pathway and the anthocyanin-specific pathway (Hyun et al., 2014). To determine the relationship between anthocyanin quantities and the expression patterns of anthocyanin biosynthesis-related genes, the expression patterns of chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX) during fruit ripening were analyzed by qRT-PCR. As shown in Fig. 1B, the expression levels of *CHI1*, *CHI2* and *F3H*, belonging to the early flavonoid pathway, and *DFR* and *LDOX*, involved in the anthocyanin-specific pathway, increased during ripening process. This suggests that the accumulation of anthocyanins occurs because of increased transcriptional levels of anthocyanin-related genes in ginseng berry, similar to those described for other plants (Hyun et al., 2014; Fang et al., 2016).

Antioxidant activities of ginseng berry at various stages of maturation

Antioxidants are molecules that protect organisms from the damage caused by unstable molecules known as reactive oxygen species; these have been implicated in the development of aging as well as various diseases including cancer, inflammation, respiratory diseases, cardiovascular diseases, neurodegenerative disorders and digestive diseases (Liu et al., 2018). Synthetic antioxidants are widely used in the food industry, as well as in other industries, including cosmetics, pharmaceutical and animal nutrition. Nevertheless, natural antioxidants, including vitamins, flavonoids and anthocyanins have been increasingly used because of concerns over the long-term safety of synthetic antioxidants (Caleja et al., 2017). The variation of these compounds during the ripening process correlated with the antioxidant activity of the fruit (Gull et al., 2012). In the case of ginseng berry, the highest DPPH free radical scavenging activity was observed in Go-S3 (RC_{50} = 658.7 μg/ml), followed by Go-S2 (RC_{50} = 946.8 μg/ml) (Fig. 2A). To further characterize the variation of antioxidant activity of ginseng berry during the ripening process, the reducing power and ORAC values of ginseng berry extracts were analyzed. As shown in Fig. 2B, the reducing potential of each extract was found to increase in a dose-dependent manner. As expected, Go-S3 (OD_{750} value = 0.15) showed the highest reducing power, followed by Go-S2 (OD_{750} value = 0.11) and Go-1 (OD_{750} value = 0.09). Similarly, Go-S3 at 50 μg/ml exhibited hydrophilic oxygen radical scavenging activity (ORAC value) of 142.1 μM TE and Go-S1 displayed the lowest ORAC value of 112.1 μM TE (Fig. 2C).

To analyze the correlation between the phytochemical content and antioxidant activity of ginseng berry, the Pearson correlation coefficient was employed. DPPH free radical scavenging activity, reducing power and ORAC values highly and positively correlated with TFC, TPC and TAC, while TCC negatively correlated with antioxidant activities (Tab. S3). Polyphenol compounds, including anthocyanins, have the potential to prevent various diseases caused by oxidative stress, and possess phenolic hydroxyl groups that are capable of donating hydrogen atom electrons to free radicals and aromatic rings that stabilize and delocalize the unpaired electrons via conjugated aromatic systems (Dai and Mumper, 2010). This suggests that the antioxidant activity of ginseng berry extracts is due to the reaction of polyphenolic compounds with free radicals, thereby converting them into more stable products.

Anti-melanogenic potential of ginseng berries

To investigate the effect of ginseng berry extracts on melanin production, the inhibitory effects of each extract on α-MSH-induced melanin production in B16F10 cells were determined. As shown in Fig. 3A, Go-S3 (100 μg/ml) significantly inhibited the α-MSH-induced melanin production in B16F10 cells, whereas Go-S1 and Go-S2 exhibited no or low inhibitory effect on α-MSH-induced melanin synthesis. Go-S3 markedly inhibited α-MSH-induced melanin synthesis in a dose-dependent manner. Go-S3 at 200 μg/ml inhibited melanin production by greater than 50% over the level generated by mock control (Fig. 3B). To investigate whether the inhibitory effects on α-MSH-induced melanin production were mediated by

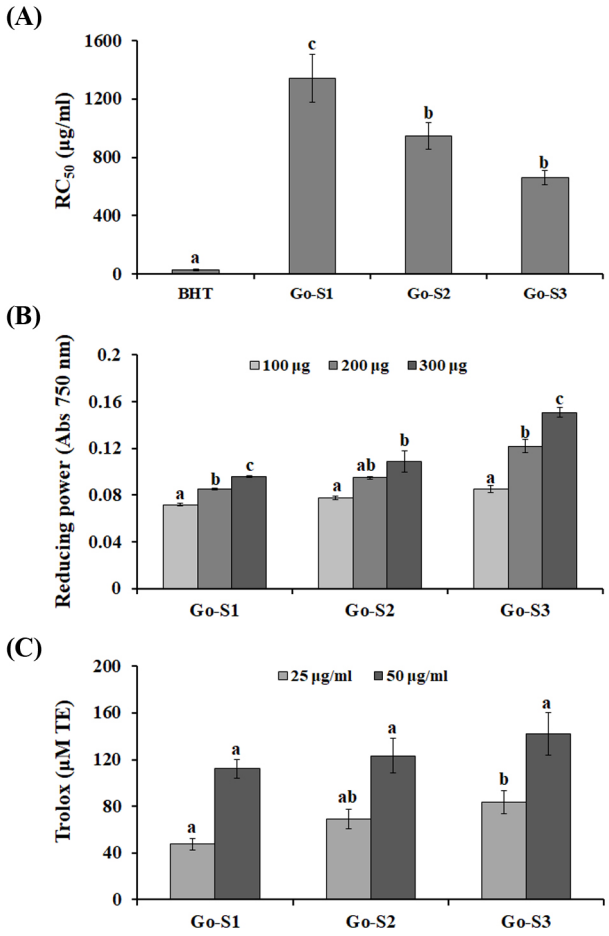

Fig. 2: Antioxidant activities of *Panax ginseng* berry extracts were measured by DPPH free radical scavenging **(A)**, reducing power **(B)** and ORAC **(C)** assays. DPPH radical scavenging activity was calculated as RC_{50}. ORAC values of each organ extract are expressed as μmol of Trolox equivalents per g dry weight. Bars represent the mean ± S.E. of three independent experiments. Different letters in each column represent significant differences between the ripening stages ($p < 0.05$).

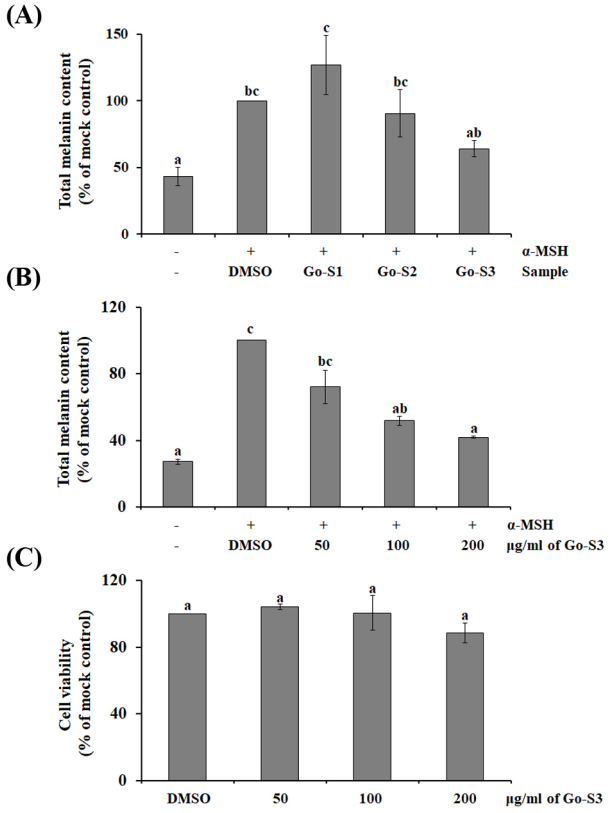

Fig. 3: Anti-melanogenic activity of *Panax ginseng* berry extracts. **(A)** Effect of *Panax ginseng* berry extracts on melanin production was analyzed in α-MSH-stimulated B16F10 cells. **(B)** Dose-dependent anti-melanogenic effects of the extract obtained from fully-ripe berries of *Panax ginseng* (Go-S3) in α-MSH-stimulated B16F10 cells. **(C)** Effect of Go-S3 on cell viability of B16F10 cells. Values are the mean ± S.E. of triplicate experiments. Bars in the same sub-figure with the same lowercase letter are not significantly different ($p < 0.05$).

cell viability, the cytotoxic effect of Go-S3 on B16F10 cells was determined by MTT assay. Cell viability at 200 μg/ml of Go-S3 was 88.5% that of mock control; however, no statistically significant change in cell viability was observed between mock control and 200 μg/ml of Go-S3 (Fig. 3C), suggesting that the inhibitory effect of Go-S3 on melanin production is not accounted for by cytotoxicity. Melanin biosynthesis is catalyzed by three melanocyte-specific enzymes, tyrosinase, Trp-1 and Trp-2 that are controlled by microphthalmia-associated transcription factor (MITF) (D'Mello et al., 2016). Inhibition of MITF-mediated gene expression can serve as a potential mechanism of action of anti-melanogenic agents. However, Go-S3 had no effect on the α-MSH-induced increases in MITF-mediated gene expression (Fig. 4A). Furthermore, the increased levels of tyrosinase activity in response to α-MSH were not suppressed by Go-S3 treatment (Fig. 4B), suggesting that Go-S3 does not regulate the transcriptional and translational machinery related to melanocyte-specific enzymes as well as MITF in B16F10 cells.

Melanin production is regulated by tyrosinase activity, as well as the expression of related genes (Slominski et al., 2004). In particular, polyphenols such as quercetin and myricetin have been found to act as competitive inhibitors of oxidation of L-DOPA by tyrosinase, thereby exhibiting whitening activity (Chang, 2009). Therefore, we hypothesized that the anti-melanogenic effect of Go-S3 was due to direct inhibition of tyrosinase activity. To test this hypothesis, we performed a tyrosinase inhibition assay in cell-free conditions. As shown in Fig. 5, the residual tyrosinase activity was 92.9%, 79.9% and 62.7% of control for 50, 100 and 200 μg/ml of Go-3, respectively, suggesting that Go-S3 directly inhibited L-DOPA oxidation activity of tyrosinase, thereby inhibiting melanin production in α-MSH-stimulated B16F10 melanoma cells.

In silico molecular docking analysis of anthocyanin compounds as tyrosinase inhibitors

Anthocyanins act as pigments in plants, but have several health benefits, including antioxidative and antimicrobial effects, anti-angiogenesis, anti-cancer, anti-diabetes, anti-obesity and neuroprotective effects as well (Putta et al., 2018). In the case of ginseng berry, anti-melanogenic activity highly correlated with the contents of polyphenolic compounds, including TAC (Tab. S3). Similarly, anthocyanins and anthocyanin-enriched fractions have been reported to suppress melanin production by inhibiting the enzymatic activity of tyrosinase; because of this property, they have become important sources of functional cosmetics, foods and pharmaceuticals (Kubota et al., 2014; Jhan et al., 2016). Molecular docking has been used as a crucial tool for analyzing the interactions between the target protein and a small molecule, and contributed to discover high-quality enzyme inhibitors that have been advanced to clinical trials (Kumalo et al., 2015). To investigate the potential mechanisms of the inhibi-

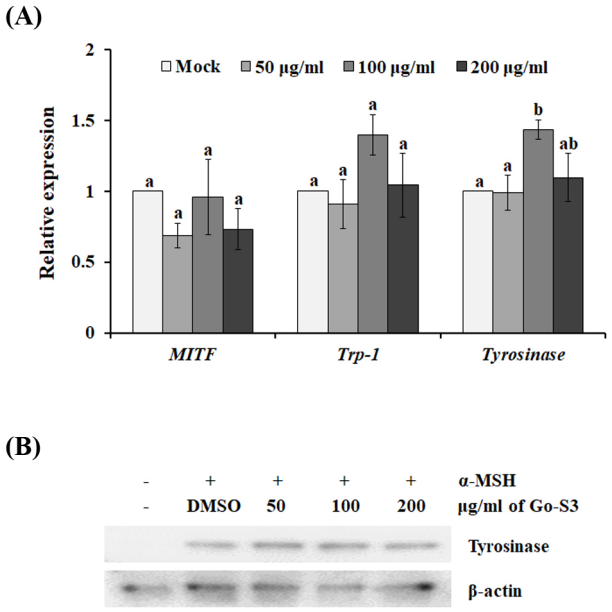

Fig. 4: Effect of *Panax ginseng* berry extract (Go-S3) on the levels of melanogenesis-related genes **(A)** and protein levels of tyrosinase **(B)** in α-MSH-stimulated B16F10 cells. The results shown in **(A)** were normalized to *β-actin* mRNA levels. **(B)** Total cell lysates were extracted and assayed by western blotting using antibody against tyrosinase. The value for the non-stimulated cells was set to 1.0. Values with the same letter are not significantly different as determined by Duncan's multiple range tests.

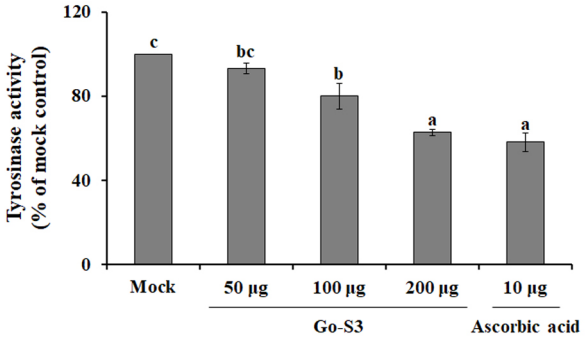

Fig. 5: Effects of *Panax ginseng* berry extract (Go-S3) on tyrosinase activity. Values are the average of triplicate experiments and are represented as the mean ± S.E.

tion of tyrosinase activity by anthocyanins, we analyzed the interaction sites and patterns between anthocyanins and tyrosinase using the structure-based molecular docking approach. We first used blind molecular docking to search for the binding site of anthocyanins to tyrosinase (Tab. S4). Then, the binding energy of D3G, C3G, and Pt3G to tyrosinase was predicted by defined molecular docking with a grid laid around tyrosinase residues found by blind docking. C3G (-5.8±0.01 kcal/mol) had higher binding affinity to tyrosinase than did Pt3G (-5.68±0.015 kcal/mol) or D3G (-5.23±0.15 kcal/mol) (Tab. 3). Tyrosinase (PDB code: 3NQ1) from *Bacillus megaterium* has a conserved active site containing six copper-coordinating histidines (His 42, His 60, His 69, His 204, His 208 and His 231) and second shell residues (Met 61, Met 184, Phe 197 and Asn 205) involved in coppe uptake and enzyme activity (KANTEEV et al., 2013). Kojic acid (-5.5 kcal/mol), the most commonly used skin-whitening agent, interacts with Phe 197, Pro 201, Asn 205 and Arg 209 residues (the entrance to the active site) of *B. megaterium* tyrosinase (DERI et al., 2016). In the case of anthocyanins, D3G, C3G and Pt3G exhibited hydrogen bond interactions with His 42 and hydrophobic interactions with His 60 and His 208 residues in active site and hydrophobic interactions with Phe 197 and Asn 205 in the second shell residues (Tab. 3). These results suggest that anthocyanin, including D3G, C3G and Pt3G, directly inhibits tyrosinase activity by interacting with copper-coordinating histidines and second shell residues.

Conclusions

We analyzed variations in antioxidant activity and anti-melanogenic potential during three ripening stages of ginseng berry. During the ripening process, antioxidant and whitening activities mediated by polyphenolic compounds such as anthocyanins increased, suggesting that the maturity of fruits has profound effects on the pharmaceutical value of ginseng berry. *In silico* molecular docking analysis suggested that C3G, D3G and Pt3G inhibit tyrosinase activity, probably by virtue of their ability to bind at active site and second shell residues of the enzyme. Nevertheless, several issues, including safety, quality and efficacy of ginseng berry, still need to be addressed. Such information will be useful for further studies on ginseng berry for its applications in pharmaceutical industries and phytocosmetics.

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Tab. 3: Defined molecular docking of anthocyanins to tyrosinase.

Compounds	Lowest binding energy (kcal/mol)	Mean binding energy (kcal/mol)	Residues involved hydrogen bond interaction	Residues involved in hydrophobic interaction	Pki (μM)[a]
Cyanidin-3-glucoside (C3G)	-5.8±0.01	-4.675±0.035	HIS 42,VAL 218	HIS 60, PHE 197, HIS 204, ASN 205, HIS 208, ARG 209, MET 215, GLY 216, VAL 217, ALA 221	55.92±0.95
Delphinidin-3-glucoside (D3G)	-5.23±0.15	-3.955±0.125	HIS 42,VAL 218	HIS 60, PHE 197, ASN 205, HIS 208, ARG 209, MET 215, GLY 216, VAL 217, ALA 221	151.685±38.01
Petunidin-3-glucoside (Pt3G)	-5.68±0.015	-4.643±0.142	HIS 42,VAL 218	HIS 60, MET 61, PHE 197, PRO 201, HIS 204, ASN 205, HIS 208, ARG 209, MET 215, GLY 216, VAL 217, ALA 221	68.69±1.945

[a] Pki indicates predicted inhibitory activity

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ORCID
Ick-Hyun Jo https://orcid.org/0000-0002-5772-8014

Address of the corresponding author:
Dr. Ick Hyun Jo, Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong 369-873, Republic of Korea
E-mail: intron@korea.kr
Dr. Tae Kyung Hyun, Department of Industrial Plant Science and Technology, College of Agricultural, Life and Environmental Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea
E-mail: taekyung7708@chungbuk.ac.kr

Supplementary material

Tab. S1: Primer sequences for qReal-time PCR analysis.

Source	Primer name	Sequence (5'-3')
B16F10 cell	MITF-F	GGCCAAGGCAGAGCAACTT
	MITF-Rev	GCCCATGGTGGCAAGCT
	TRP1-F	GAGTGACATCCTGTGGCTCA
	TRP1-Rev	CGATACCCTGGGAACACTTT
	Tyrosinase-F	ATAGGTGCATTGGCTTCTGG
	Tyrosinase-Rev	CCAACGATCCCATTTTTCTT
	-actin-F	CCCACTCCTAAGAGGAGGATG
	-actin-Rev	AGGGAGACCAAAGCCTTCAT
Panax ginseng	PgCHI1-F	TCAAACCTTCCAACCTGGCT
	PgCHI1-Rev	GAGACACCATGCTTGCCAAT
	PgCHI2-F	TCGTTCACTTCTTCCAGTCCA
	PgCHI2-Rev	CCACCCAGGTACCACTTCTT
	PgF3H-F	CTATCGGAGGCAATGGGTCT
	PgF3H-Rev	ACCAACCTGATCCTGAAGCA
	PgDFR-F	CGACGAAACCAGTTGGAGTG
	PgDFR-Rev	GCGGCAATGTTGGCATGATA
	PgLDOX-F	TTGTGGCCAACTTGAATGGG
	PgLDOX-Rev	GGCCTCTTAGCTCCTTAGCA
	PgActin-F	CCCGAGAGAAAGTATAGTGTATGGA
	PgActin-Rev	TAGAGCTCTTCAACAACCACTTTTT

Tab. S2: Gradient conditions for HPLC analysis.

Analysis	Step	Time (min)	Solvent A (%)	Solvent B (%)
Anthocyanin	0	Initial	95[a]	5[b]
	1	0.30	95[a]	5[b]
	2	6.00	80[a]	20[b]
	3	8.00	5[a]	95[b]
	4	10.00	95[a]	5[b]
	5	13.00	95[a]	5[b]
Saponin	0	Initial	82.0[c]	18.0[b]
	1	20.0	82.0[c]	18.0[b]
	2	30.0	70.0[c]	30.0[b]
	3	50.0	50.0[c]	50.0[b]

[a] 1% Phosphoric acid in water
[b] Acetonitrile
[c] Water

Tab. S3: Correlations between the biological activities and phytochemical contents of ginseng berries.

	TPC	TFC	TSC	TCC	TAC	DPPH	Reducing Power	ORAC	Anti-melanogenic
TPC	1	-0.913	0.032	-0.911	0.911	-0.998	0.996	0.996	-0.992
TFC		1	-0.378	-0.664	1.000	-0.886	0.992	0.875	-0.957
TSC			1	-0.441	-0.383	-0.094	-0.260	0.117	0.092
TCC				1	-0.660	0.935	-0.752	-0.943	0.853
TAC					1	-0.884	0.992	0.872	-0.955

Tab. S4: *In silico* blind molecular docking of anthocyanins to tyrosinase.

Compounds	**Lowest binding energy (kcal/mol)**	**Mean binding energy (kcal/mol)**	**Number of residues involved in hydrophobic interactions**	**Pki (µM)**
Cyanidin-3-glucoside (C3G)	-5.24	-4.34	13	144.8
Delphinidin-3-glucoside (D3G)	-2.37	-2.37	7	18230
Petunidin-3-glucoside (Pt3G)	-3.3	-3.3	7	3790