Downregulation of Melanin Synthesis by Haginin A and Its Application to *In Vivo* Lightening Model

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Haginin A, an isoflav-3-ens isolated from the branch of *Lespedeza cyrtobotrya*, is almost unknown. Here, we report that haginin A exhibits a strong hypopigmentary effect in Melan-a cells and significantly inhibits melanin synthesis. Haginin A shows potent inhibitory effects with an IC_{50} (half-maximal inhibitory concentration) value of 5.0 μM on mushroom tyrosinase activity, and functioned as a noncompetitive inhibitor. Also, haginin A decreased microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related protein-1 (TRP-1) protein production. To identify the signaling pathway of haginin A, the ability of haginin A to influence extracellular signal-regulated protein kinase (ERK) and Akt/protein kinase B (PKB) activation was investigated. Apparently, haginin A induced ERK and Akt/PKB in a dose-dependent manner. In addition, the specific inhibition of the ERK and the Akt/PKB signaling pathways by PD98059 and LY294002, respectively, increased melanin synthesis. Furthermore, haginin A decreased UV-induced skin pigmentation in brown guinea-pigs. Also, haginin A presented remarkable inhibition on the body pigmentation in the zebrafish model system and decreased tyrosinase activity. Together, haginin A is an effective inhibitor of hyperpigmentation caused by UV irradiation or by pigmented skin disorders through downregulation via ERK and Akt/PKB activation, MITF, and also by the subsequent downregulation of tyrosinase and TRP-1 production.

Journal of Investigative Dermatology (2008) **128,** 1227–1235; doi:10.1038/sj.jid.5701177; published online 22 November 2007

INTRODUCTION

In mammals, melanin production is restricted to the melanocytes of the skin, hair follicles, and pigment epithelium in the retina. Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 are involved in melanogenesis, in which tyrosine is converted into melanin pigments (Kobayashi *et al.*, 1994; Ando *et al.*, 1999). In particular, tyrosinase plays a key role in this process, because it catalyzes two rate-limiting reactions involved in melanogenesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the oxidation of DOPA, which results in the formation to dopaquinone (Hearing and Tsukamoto, 1991).

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; ERK, extracellular signal-regulated protein kinase; MEK, MAPK/ERK kinase; MITF, microphthalmia-associated transcription factor; PKB, protein kinase B; PTU, 1-phenyl-2-thiourea; TRP, tyrosinase-related protein

Received 6 March 2007; revised 8 August 2007; accepted 20 September 2007; published online 22 November 2007

Several of the known natural melanin synthesis inhibitors, including arbutin and kojic acid, have already been the focus of studies, and are currently being utilized as cosmetic additives (Fujimoto *et al.*, 1998). However, it is clearly necessary to find safer and more effective skin-whitening agents, due to the carcinogenic potential of kojic acid, not to mention its weak whitening effects. A great deal of attention has continuously focused on the application of natural products in the cosmetics industry (Shimizu *et al.*, 2003; Park *et al.*, 2004). The signaling pathways responsible for the regulation of melanocyte differentiation have yet to be clearly elucidated, although several important advances have been made in this regard.

Microphthalmia-associated transcription factor (MITF), characterized by an essential basic helix–loop–helix leucine zipper structure, is currently believed to regulate melanocyte pigmentation, proliferation, and survival (Steingrimsson *et al.*, 1994). In addition, MITF has been shown to effectively transactivate the tyrosinase, TRP-1, and TRP-2 melanogenic genes *in vitro*, via binding to an M-box motif that is present in the promoters of these genes (Yasumoto *et al.*, 1997; Bertolotto *et al.*, 1998). The use of molecules that can trigger the signaling pathways upstream of these key melanocyte genes might provide a useful tool in furthering our current understanding of melanocyte differentiation.

In this study, we have screened a large number of plant extracts using Melan-a cells and we found that haginin A, which had been isolated from the *Lespedeza cyrtobotrya* branch, exerted a profound inhibitory effect on the synthesis of melanin. However, the biological effects of haginin A

are comparatively unknown. Therefore, we investigated the effects of haginin A on melanogenesis in Melan-a cells with regard to extracellular signal-regulated protein kinase (ERK) and Akt/protein kinase B (PKB) pathway activation.

RESULTS

Inhibitory effects of haginin A on tyrosinase activity and melanin biosynthesis of *Streptomyces bikiniensis*

The structure of haginin A is shown in Figure 1. The effects of haginin A on tyrosinase activity were determined via mushroom tyrosinase assays. The IC_{50} (half-maximal inhibitory concentration) values of a variety of tyrosinase inhibitors were determined using L-tyrosine as a substrate. Haginin A, arbutin, and kojic acid were all shown to inhibit mushroom tyrosinase, and all of these compounds exerted their effects in a dose-dependent manner. Among these, haginin A was determined to exert the most profound inhibitory effects on mushroom tyrosinase ($IC_{50} = 5\,\mu M$), followed by kojic acid ($IC_{50} = 50.1\,\mu M$) and arbutin ($IC_{50} = 58.4\,\mu M$) (Table 1). Moreover, haginin A was 10-fold more potent than kojic acid in its inhibitory effects on tyrosine oxidation. These inhibitory effects on tyrosinase activity and subsequent melanin formation were also confirmed in *S. bikiniensis*. Apparently, haginin A inhibited the biosynthesis of melanin in *S. bikiniensis* (Table 1). We also conducted a kinetic study of the catalysis of L-tyrosine oxidation by mushroom tyrosinase in the presence of haginin A (Figure 2). Haginin A was shown to reduce the maximal velocity (V_{max}) of mushroom tyrosinase activity, but no significant effects on the K_m values were observed. As summarized in Table 2, the V_{max} value (ΔA_{490} per minute) of mushroom tyrosinase activity was 3.6×10^{-2}, and the K_m value was 0.2 mM L-tyrosine. The mushroom tyrosinase activity in the presence of haginin A at 0.87 and 1.67 μM demonstrated identical K_m values (0.2 mM) and exhibited 2.6×10^{-2} and 1.7×10^{-2}, respectively, as V_{max} values. Therefore, haginin A was clearly implicated as a non-competitive inhibitor of the mushroom enzyme, with K_i values of 2.17 ± 0.48 and $1.48 \pm 0.41\,\mu M$. The profound inhibitory activity on the oxidase activities of mushroom tyrosinase by haginin A strongly suggests that haginin A might be a potential candidate for development of a skin-whitening agent.

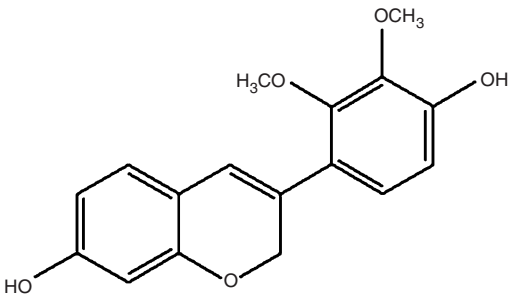

Figure 1. The structure of haginin A.

Haginin A exerts strong hypopigmentary effects on Melan-a cells

To investigate whether haginin A exerts cytotoxic effects on Melan-a cells, we applied haginin A to these cells at concentrations of 0.4–13.3 μM for 4 days, then assessed cell viability via crystal violet assays. Melan-a cells are syngeneic,

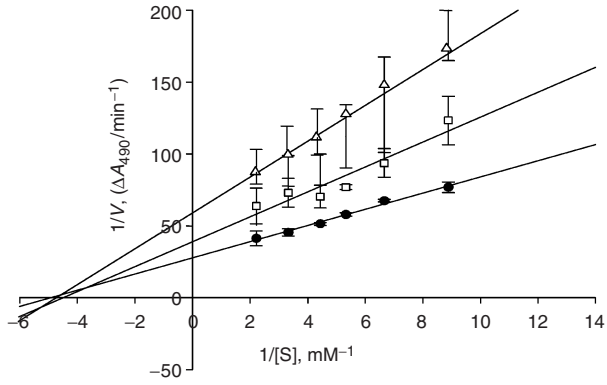

Figure 2. Lineweaver–Burk plot of mushroom tyrosinase in the presence of haginin A. Data are expressed as mean values of 1/*V*, inverse of the increase in absorbance at 490 nm minute^{-1} (ΔA_{490} per minute), and means of three independent tests with different concentrations of L-tyrosine used as a substrate. Haginin A concentrations as an inhibitor were as follows: □, 0.87 μM; △, 1.67 μM; ●, control.

Table 1. Inhibitory effects on mushroom tyrosinase activity and melanin formation in *Streptomyces bikiniensis*

Compound	Mushroom tyrosinase IC_{50} (μM)	*S. bikiniensis* NRRL B-1049 inhibition zone (mm) 30 μg	20 μg	10 μg
Haginin A	5.0 ± 0.013	42	35	23
Arbutin	58.4 ± 0.017	0	0	0
Kojic acid	50.1 ± 0.015	0	0	0
p-Methylphenol	120.0 ± 0.020	35	25	15

IC_{50}, half-maximal inhibitory concentration.
Tyrosinase was preincubated with test substances at 25 °C for 10 min prior to incubation with L-tyrosine for 30 min and the absorbance was determined at 490 nm. Each value represents the mean ± SD of three experiments.

Table 2. Kinetic parameters of mushroom tyrosinase in the presence of haginin A

Compound	K_m (M)	V_{max} (ΔA_{490} per min)	K_i (M)
None	2.03×10^{-4}	3.6×10^{-2}	—
Haginin A	2.03×10^{-4}		
(8.67×10^{-7} M)		2.6×10^{-2}	$2.17 \pm 0.48 \times 10^{-6}$
(1.67×10^{-6} M)		1.7×10^{-2}	$1.48 \pm 0.41 \times 10^{-6}$

V_{max}, maximal velocity.
The kinetic parameters were obtained with L-tyrosine as a substrate using the Lineweaver–Burk plot shown in Figure 2. K_m and K_i values are represented as the molar concentration and V_{max} values are expressed as the increases in absorbance at 490 nm.

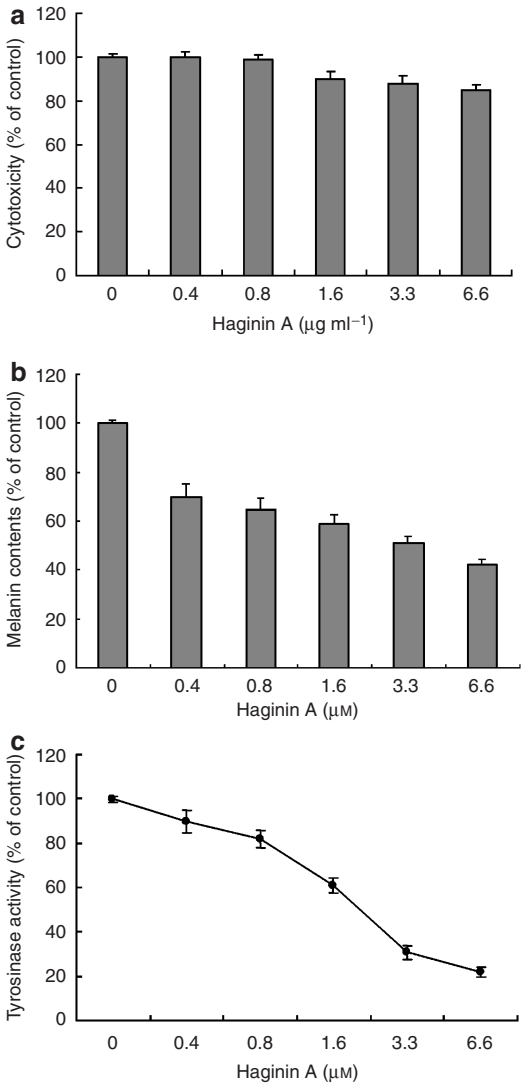

Figure 3. Effects of haginin A on melanogenesis of Melan-a cells. The cells were cultured with 0.4–6.6 μM of haginin A for 4 days. (**a**) Cytotoxicity, (**b**) melanin contents, and (**c**) tyrosinase activity were measured in Melan-a cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means ± SD.

are associated with the B16 melanoma and its sublines, provide an excellent parallel non-tumorigenic cell line for examining the malignancy of melanoma, and have similar phenotype as the primary mouse melanocyte (Bennett *et al*, 1987). As is shown in Figure 3a, haginin A exerted no cytotoxic effects on the Melan-a cells at the tested concentrations. To characterize the effects of haginin A, we measured melanin contents of Melan-a and HEMn (human epidermal melanocytes) cells. We used 1-phenyl-2-thiourea (PTU) and kojic acid as positive controls in these studies because of their known inhibitory effects on melanin synthesis. The levels of melanin in Melan-a and HEMn cells were determined to have been significantly reduced as a result of haginin A treatment. As shown in Table 3, the concentration of haginin A required for a 50% inhibition in Melan-a and HEMn cells was found to be approximately 3.3 and 2.7 μM, respectively. However, kojic acid was shown to exert no detectable effects on melanin production in Melan-a and HEMn cells.

Table 3. Effects of haginin A, PTU, and kojic acid on melanin production of Melan-a and HEMn cells

	Melanin synthesis IC_{50} (μM)		Cytotoxicity LD_{50} (μM)	
Compound	Melan-a	HEMn	Melan-a	HEMn
Haginin A	3.3	2.7	14.5	11.8
PTU	6.6	8.6	>200	>200
Kojic acid	>200	>200	>200	>200

IC_{50}, half-maximal inhibitory concentration; LD_{50}, dose lethal to 50% of animals tested; PTU, 1-phenyl-2-thiourea.

Melanin levels were significantly reduced in a dose-dependent manner by haginin A treatment in Melan-a cells (Figure 3b). In addition, haginin A at the same concentrations also decreased tyrosinase activity in Melan-a cells (Figure 3c). These findings suggest that haginin A regulates tyrosinase and subsequently suppresses melanin synthesis in Melan-a cells.

Haginin A reduces tyrosinase protein levels and reduces MITF protein levels

Melanogenesis is known to be controlled by an enzymatic cascade, which is regulated at the levels of tyrosinase, TRP-1, and TRP-2 (Gaggioli *et al*., 2003). The amounts of those enzymes in the cells during the reduced pigmentation elicited by haginin A were determined via western blot analysis. Haginin A suppressed the expression levels of tyrosinase and TRP-1 protein expression in Melan-a cells, whereas there was no effect on TRP-2 levels in these cells (Figure 4).

In particular, the induction level of tyrosinase was higher than that of TRP-1. The amount of TRP-2 remained constant throughout the course of the experiment, thus working as an appropriate internal control.

MITF is reported to bind to the M-box within the tyrosinase promoter and thus to regulate tyrosinase gene expression (Bentley *et al*., 1994). As MITF controls tyrosinase protein levels, we examined the level of MITF protein after haginin A treatment. As shown in Figure 5a, MITF protein levels were reduced in a dose-dependent manner. Since a decreased MITF gene expression may be responsible for a diminished level of MITF protein, we next examined whether haginin A has an effect on MITF transcription.

Reverse transcription PCR assays using MITF-specific primers produced a 366 bp fragment corresponding to the MITF mRNA. However, we did not observe any significant change of the level of this PCR fragment in haginin A-treated cells (Figure 5b), whereas the levels of MITF protein reduced significantly (Figure 5a). Accordingly, we suggest that the MITF protein reduction by haginin A may be due to MITF degradation, not to suppression of MITF gene expression.

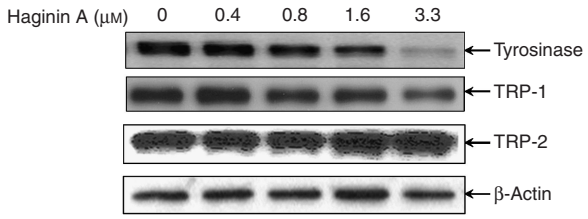

Figure 4. Haginin A attenuates the levels of tyrosinase and TRP-1 but has no effect on TRP-2. Melan-a cells were treated with 0.4–3.3 μM of haginin A for 4 days. Whole-cell lysates were then subjected to western blot analysis using antibodies against tyrosinase, TRP-1, and TRP-2. Equal protein loadings were confirmed using anti-actin antibody.

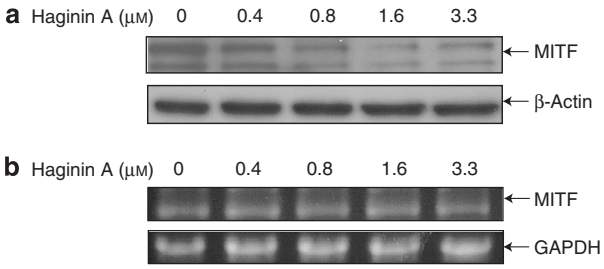

Figure 5. Effects of MITF gene expression by haginin A in Melan-a cells. (**a**) Inhibition of MITF protein expression by haginin A. Melan-a cells were treated with the indicated concentrations of haginin A. Whole-cell lysates were subjected to western blot analysis using MITF. Equal protein loadings were confirmed using anti-actin antibody. (**b**) The effects of haginin A on the MITF mRNA level with indicated concentrations of haginin A. Total RNA was isolated from the cells, and the cDNA was prepared. Equivalent amounts of cDNA were amplified with primers specific for MITF, and GAPDH primers were used as a control to ensure the even loading of the target cDNA. The resulting PCR products were analyzed by agarose gel electrophoresis.

Haginin A induces phosphorylation of ERK and Akt/PKB in Melan-a cells

To elucidate the mechanisms underlying the hypopigmentary effects exerted by haginin A, we attempted to characterize the changes in melanogenesis-related signals induced by haginin A via western blot analysis. Furthermore, we examined whether haginin A is able to influence ERK and/or Akt/PKB activation. As shown in Figure 6, haginin A was shown to elicit ERK phosphorylation, and this effect was exerted in a dose-dependent manner. Also, phosphorylation of Akt/PKB was much more activated.

We also investigated events upstream of ERK and found that haginin A stimulated the phosphorylation of MEK (MAPK/ERK kinase). The kinetics of MEK and ERK activation after haginin A stimulation showed similar patterns. In addition, we examined cyclic AMP response element binding protein, which is known to activate the MITF promoter. However, haginin A had no effects on its phosphorylation level. These results indicate that haginin A induces the phosphorylation of ERK and Akt/PKB in a dose-dependent manner.

Effect of PD98059 and LY294002 on melanin synthesis in Melan-a cells

Since the phosphorylation of ERK and Akt/PKB seemed to decrease melanin synthesis, we examined whether the ERK

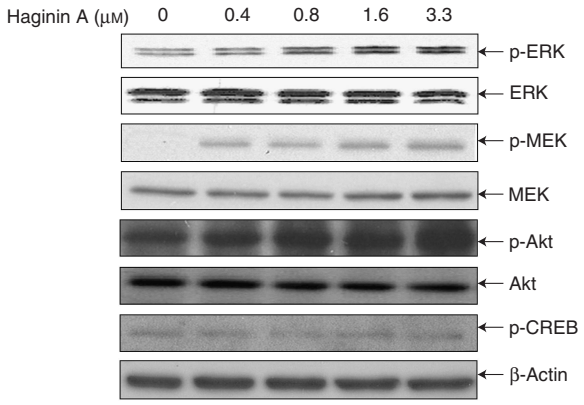

Figure 6. Haginin A induces ERK and Akt/PKB phosphorylation by haginin A in Melan-a cells. The Melan-a cells were treated with the indicated concentrations of haginin A. Whole-cell lysates were then subjected to western blot analysis using antibodies against phospho-specific ERK (p-ERK), phospho-specific MEK (p-MEK), phospho-specific Akt (p-Akt), and phospho-specific cyclic AMP response element binding protein (p-CREB). Equal protein loading was confirmed by reaction with actin, phosphorylation-independent ERK, Akt, MEK antibodies.

and/or the Akt/PKB signaling pathways were involved in melanogenesis. Therefore, we investigated melanin synthesis after treating with PD98059, a selective inhibitor of MEK that is a specific upstream activator of ERK, to study the role of ERK in the haginin A-induced inhibition of melanin production. We also used LY294002, a phosphatidylinositol 3-kinase inhibitor, which blocks the Akt/PKB signaling pathway. As shown in Figure 7a, melanin synthesis was clearly induced after treatment with PD98059, but LY294002 promoted melanogenesis less than PD98059. Next, we wanted to determine whether PD98059 inhibits ERK and LY294002 inhibits Akt/PKB activation in Melan-a cells, and indeed PD98059 was found to block ERK signaling in haginin A-treated cells and in normal cells, indicating that the inhibition of the ERK pathway induces melanin synthesis and LY294002 also blocks Akt/PKB slightly (Figure 7b). These results suggest that the ERK and Akt/PKB pathway plays a critical role in melanogenesis.

Effects of haginin A on UVB-induced hyperpigmentation in brown guinea-pig skin

The whitening effect of haginin A was examined using a UVB-induced hyperpigmentation model in brown guinea-pigs. Figure 8a shows a photograph of the whitening effects on the guinea-pig dorsal skin. Haginin A was topically applied to the UV-induced hyperpigmented dorsal skin areas twice a day for 4 weeks from the day after the last tanning. A visible decrease in hyperpigmentation was observed 2 weeks after the treatment with haginin A, when compared to the vehicle group. The degree of pigmentation decreased (ΔL-value) before and 4 weeks after the application of haginin A was 3.4. In addition, a strongly visible decrease in hyperpigmentation was observed at 4 weeks after treatment with haginin A compared to the vehicle control group (Figure 8c).

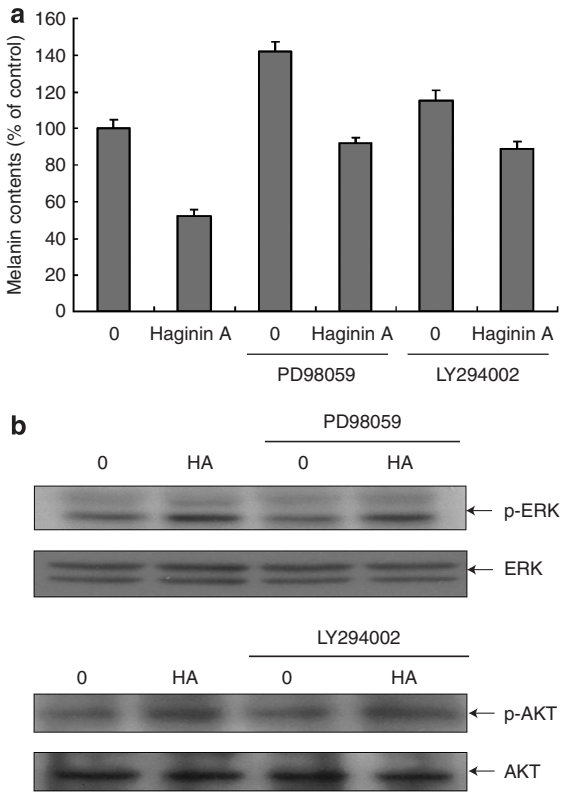

Figure 7. Effects of PD98059 and LY294002 by haginin A on melanogenesis in Melan-a cells. Cells were pretreated with 20 μM of PD98059 for 1 hour and then cultured with 3.3 μM of haginin A for 4 days. (**a**) Melanin content was measured as described in Materials and Methods. Each column shows the mean ± SD of triplicate determinations. (**b**) Cells were cultured with 3.3 μM of haginin A in the absence or presence of 20 μM PD98059 and LY294002. Whole-cell lysates were then subjected to western blot analysis with antibodies against phospho-specific ERK (p-ERK) and phospho-specific Akt (p-Akt). Equal protein loading was confirmed by reaction with actin and phosphorylation-independent ERK, Akt antibodies.

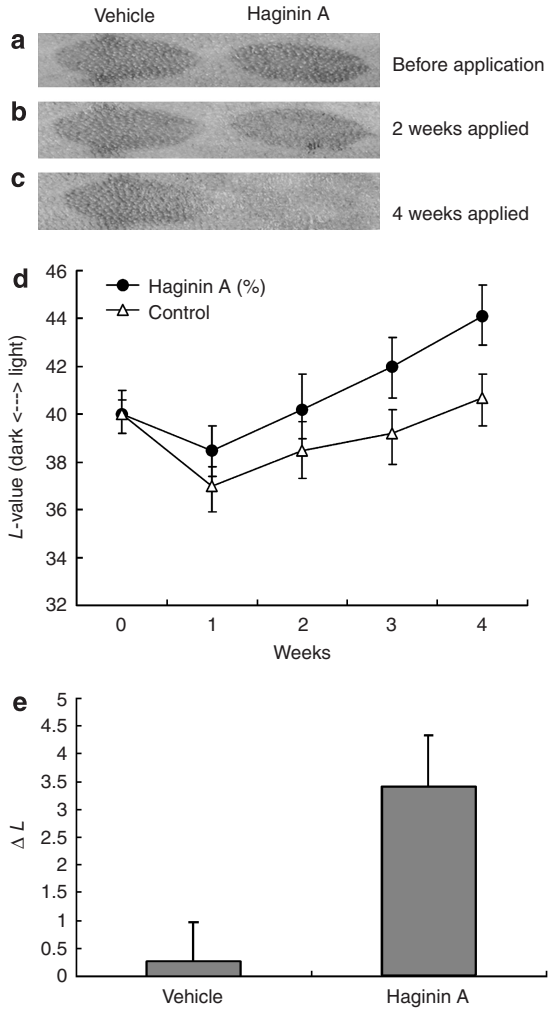

Figure 8. Effect of haginin A on UVB-induced hyperpigmentation in guinea-pig skin. (**a**–**c**) Representative photographs showing the lightening effects of haginin A on UVB-induced hyperpigmentation; all photographs are from the same animal ((**a**) initial pigmentation after UV irradiation but before the application of haginin A, (**b**) 2 weeks, (**c**) 4 weeks). The vehicle did not affect the skin color compared with the control. (**d**) Changes of *L*-value after the daily topical applications of the vehicle and haginin A. (**e**) The degree of pigmentation (Δ*L*-value) before and 4 weeks after daily topical applications of the vehicle and haginin A. The data are expressed as a mean Δ*L*-value ± SD. The Δ*L*-value was measured using a chromameter. Groups of four animals were used in this experiment.

Effects of haginin A on tyrosinase activity in zebrafish

Phenotype-based evaluation of candidate compounds gives robust and reliable data for a further experiment. Zebrafish has melanin pigments on the surface, allowing simple observation on the pigmentation process without complicated experimental procedures.

Therefore, we investigated the effects of haginin A on the pigmentation of zebrafish. As a positive control, we used PTU, a sulfur-containing tyrosinase inhibitor, which is used widely in zebrafish research (Elsalini and Rohr, 2003).

As shown in Figure 9a, 4 μM haginin A produced remarkable inhibition on the body pigmentation. Also, we measured the tyrosinase activity using the whole extract of zebrafish. There was a substantial decrease in tyrosinase activity after treatment with haginin A. As expected, haginin A remarkably decreased tyrosinase activity, similar to PTU (Figure 9c).

DISCUSSION

Haginin A was isolated initially in 1980, as a metabolite of *L. cyrtobotrya* (Toshio *et al.*, 1980). The *Lespedeza* species (Leguminosae) are known to produce a number of unique isoflavonoids, and have also been determined to possess antioxidant activity (Miyase *et al.*, 1999). However, little is known about biological activities of 7,4′-dihydroxy-2′, 3′-dimethoxyisoflavene, which is also commonly referred to as haginin A. In this study, we found that haginin A inhibits mushroom tyrosinase activity in a noncompetitive inhibitory manner with the same K_m value.

Melanogenesis is a major function of melanocytes. To investigate the effect of haginin A on melanin production, we treated melanocytes with haginin A and observed a dramatic inhibition of melanin content upon increasing the concentration of haginin A in the culture medium. We then compared the effects of haginin A with those of PTU and kojic acid as positive

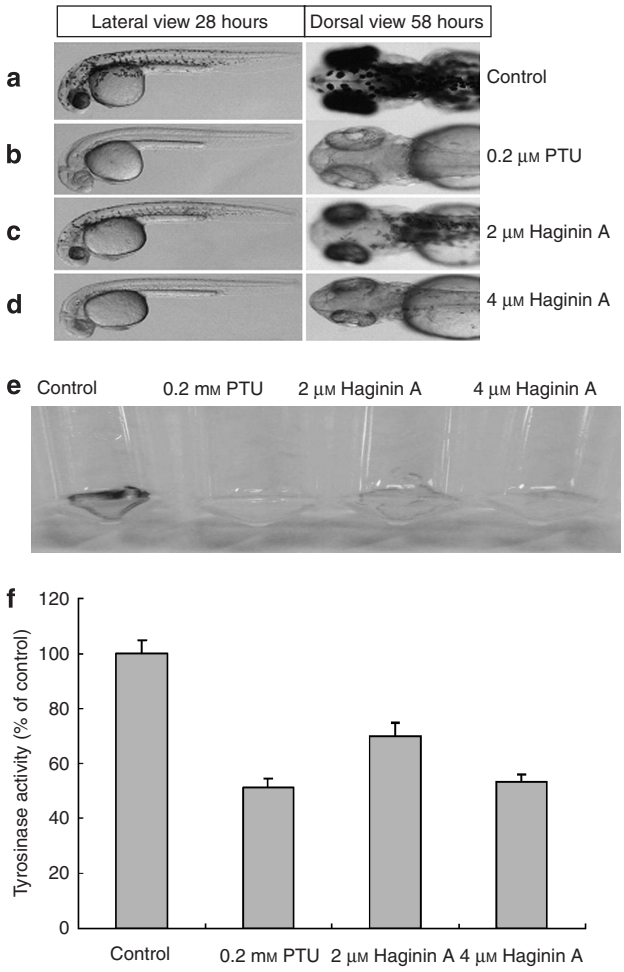

Figure 9. Effects of haginin A on melanogenesis in zebrafish.
(**a**–**d**) Representative photographs of zebrafish. Synchronized embryos were treated with melanogenic inhibitors as the indicated concentrations. The compounds were dissolved in 0.1% DMSO, then added to the embryo medium. The effects on the pigmentation of zebrafish were observed under the stereomicroscope ((**a**) untreated zebrafish embryo, (**b**) 0.2 mM PTU, (**c**) 2 μM haginin A, (**d**) 4 μM haginin A). (**e**) Melanin pigment. About 100 embryos were collected and dissolved in lysis buffer. After centrifugation, melanin pigment (upper panel) was redissolved in 1 N NaOH (lower panel). (**f**) Tyrosinase activity. For measurement of tyrosinase activity, 250 μg of total protein was incubated with L-DOPA (final 0.5 mM), and then quantified using a spectrometer. The results are shown as a percentage of control. All experiments were repeated three times.

controls in these trials because of their known inhibitory effects on melanin synthesis (Mishima *et al.*, 1988; Elsalini and Rohr, 2003). Our results show that the inhibitory effect of haginin A on melanin was stronger than that of kojic acid.

To study the action of haginin A on melanogenesis, we investigated the effect of haginin A on tyrosinase activity. Haginin A significantly inhibits tyrosinase activity in a dose-dependent manner; this effect of haginin A was stronger than that of kojic acid. These results suggest that the decreased melanogenesis by haginin A could be achieved by its inhibitory action upon the signaling pathway regulating tyrosinase activity.

In this study, we investigated whether haginin A exerts hypopigmentary effects in Melan-a cells, and determined that haginin A potently induced the downregulation of melanin synthesis in these cells, ostensibly by virtue of an elicited reduction in the production of tyrosinase. Therefore, we surmise that reduced tyrosinase activity may be responsible for the low pigment content observed in cells treated with haginin A. This suggests that the reduced pigmentation seen with haginin A treatment is probably attributable to the effects of haginin A on the signaling pathways responsible for the regulation of tyrosinase.

Furthermore, our results show that haginin A reduced MITF protein levels. According to a recent study, it has been demonstrated that the phosphorylation of MITF at serine 73 is responsible for MITF ubiquitination and degradation. Also, it has been demonstrated that the inhibition of the ERK pathway by a specific inhibitor, PD98059, increased melanin synthesis (Englaro *et al.*, 1998). These findings suggest that sustained ERK activation can suppress melanogenesis via increased MITF degradation, which is induced by ERK-dependent MITF phosphorylation (Busca and Ballotti, 2000).

Previous studies have shown that ERK activation is related to cyclic AMP-induced melanogenesis in B16 melanoma cells (Englaro *et al.*, 1995). Our findings support the notion that the inhibition of the ERK pathway induces melanin synthesis; that is, ERK pathway activation may be responsible for the observed inhibition of melanogenesis by haginin A. In our experiments, haginin A obviously stimulated the phosphorylation of ERK and inhibited the synthesis of melanin in Melan-a cells. Therefore, the activation of the ERK signaling pathway might perform an important function in the regulation of melanogenesis and also in the proliferation of Melan-a cells.

In our study, we have shown that haginin A induced ERK phosphorylation and reduced melanin synthesis. Recently, Akt/PKB activation occurs via a pathway that includes phosphatidylinositol 3-kinase (Downward, 1998). Interestingly, Akt/PKB was stimulated at a later stage by haginin A in Melan-a cells. Many types of neuronal cells are known to depend on the phosphatidylinositol 3-kinase/Akt kinase pathway as a crucial growth-promoting and an antiapoptotic signal (Zhou *et al.*, 1998). Since melanocytes are derived from the neuroectoderm, it is possible that the Akt/PKB kinase pathway also plays an important role in melanocyte proliferation. Earlier studies have shown that the activation of Akt/PKB is responsible for the suppression of melanin production in G361 melanoma cells (Oka *et al.*, 2000) and that the specific inhibition of the Akt/PKB pathway by LY294002 stimulates melanin synthesis in B16 melanoma cells (Busca *et al.*, 1996). Here, we showed that haginin A induced Akt/PKB phosphorylation and inhibited melanin synthesis. Therefore, we expected that signal transduction through Akt/PKB could be related to a decrease in the melanin content. In our study, LY294002 treatment increased melanin synthesis in Melan-a cells. Thus, our findings are in agreement with previous studies, which showed that the Akt/PKB pathway might be involved in the melanogenesis of melanocytes. Moreover, PD98059 treatment,

which blocked ERK activation, increased melanin synthesis more than LY294002 treatment in Melan-a cells. In addition, the results of this study showed ERK and Akt/PKB activation by haginin A in a dose-dependent manner. Therefore, we suggest that ERK activation may cause MITF reduction, and this ultimately results in reduced melanin synthesis.

Furthermore, we observed efficient lightening effects following the topical application of haginin A to the dorsal skin of brownish guinea-pigs in which hyperpigmentation had been induced by exposure to UVB. This suggests that the lightening effects exerted by haginin A on UVB-induced hyperpigmentation may be attributable to the inhibition of melanin production in active melanocytes.

In addition, we investigated the effects of haginin A on the pigmentation of zebrafish. Zebrafish is a highly advantageous vertebrate model organism, because of its similar organ systems and gene sequences to human beings (Love *et al.*, 2004). In this study, haginin A decreased the body pigmentation and the tyrosinase activity.

One interesting finding of this study involves the consensus between the results obtained from *in vivo* and *in vitro* experiments. Haginin A was determined to be effective with regard to the amelioration and reversal of UVB-induced hyperpigmentation and zebrafish *in vivo*, and was also determined to effectively inhibit the production of melanin *in vitro*.

In summary, we have been generally successful in our attempt to characterize the hypopigmentary effects exerted by haginin A, as well as the mechanisms responsible for these effects. In this study, we report that the inhibitory effects on tyrosinase activity by haginin A isolated from the *L. cyrtobotrya* branch were more pronounced than those seen with kojic acid. Compounded with the fact that haginin A exhibited no cytotoxic activity in our study, it can be surmised that haginin A may prove quite useful as a natural depigmentation agent. Our findings also suggest that haginin A exerts an inhibitory effect on melanogenesis in Melan-a cells via downregulation of MITF and phosphorylation of ERK and Akt/PKB, which ultimately results in the inhibition of tyrosinase production. Furthermore, our findings indicate that haginin A can inhibit the synthesis of melanin via a unique dual-action mechanism. If this is the case, haginin A might have significant therapeutic value in terms of the prevention or treatment of hyperpigmentation. To further delineate the structure–activity relationships inherent to the chemical, we are attempting to synthesize haginin A with a range of haginin A derivatives, and intend to further examine the skin-whitening effects of haginin A and its derivatives in an animal model.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase, L-tyrosine, L-DOPA, 12-*o*-tetradecanoylphorbol-13-acetate, and PTU were all purchased from Sigma Chemical Co. (St Louis, MO). The plastics used in the tissue culture were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ), and the media and additives were obtained from Gibco (Grand Island, NY).

Extraction and isolation of haginin A

Haginin A used in this study was isolated from *L. cyrtobotrya*. In brief, air-dried *L. cyrtobotrya* (1 kg) was percolated with MeOH at 25 °C for 3 weeks. The residue obtained after the removal of the solvent (4 g) was diluted with H_2O (500 ml) and extracted using EtOAc (500 ml $\times$ 3). The extract left a dark syrup (1 g) upon concentration, and this was purified via Sephadex LH-20 column chromatography using a solvent system consisting of MeOH and H_2O (8:2). The final purification was accomplished via HPLC (C_{18} column) with a 30% aqueous acetonitrile (YMC-ODS-AM 250 $\times$ 6 mm, 10 μm; the flow rate, 1.5 ml minute^{-1}; detection, UV at 220 nm) solvent system. On the basis of the results of electron spray ionization–mass spectrometry, ^{1}H-NMR (nuclear magnetic resonance), and ^{13}C-NMR spectral data, the purified compound was identified as haginin A ($C_{17}H_{16}O_5$; molecular weight 300.10 kDa) (Toshio *et al.*, 1980).

Tyrosinase activity

Tyrosinase activity in Melan-a cells was determined as described previously (Busca *et al.*, 1996), with a slight modification (Park *et al.*, 2004). In brief, Melan-a cells were cultured in six-well plates. After incubated with the test substances for 4 days, the cells were washed in ice-cold phosphate-buffered saline and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. The lysates were clarified by centrifugation for 5 minutes at 10,000 *g*. After the quantification of protein levels and the adjustment of concentrations using lysis buffer, 90 μl of each lysate, contained identical amounts of protein, was placed into each 96-well plate, and 10 μl of 15 mM L-DOPA was added per well. After incubation at 37 °C, we measured absorbance every 10 minutes, for at least 1 hour, at a wavelength of 475 nm, using an ELISA reader. A cell-free assay system was used to test for the direct effects of haginin A on tyrosinase activity. The reaction mixture for the determination of mushroom tyrosinase (EC 1.14.18.1) activity contained 150 μl of 0.1 M phosphate buffer (pH 6.5), 3 μl of sample solution, 8 μl of mushroom tyrosinase (2,100 U ml^{-1}, 0.05 M phosphate buffer, pH 6.5), and 36 μl of 1.5 mM L-tyrosine in a 96-well microplate (SPL, Pocheon, Korea). Following incubation at 37 °C, absorbance was measured at 490 nm using a microplate reader (Bio-Rad 3550; Bio-Rad, Hercules, CA).

Kinetic analysis of tyrosinase inhibition by haginin A

Using various L-tyrosine concentrations as a substrate, we added mushroom tyrosinase (2,100 U ml^{-1}) and 0.1 M phosphate buffer (pH 6.5), either with or without a test sample to 96-well plates in a total volume of 200 μl as assay mixtures. The initial rate with dopachrome formed from the reaction mixture was determined as the increase of absorbance at a wavelength 490 nm minute^{-1}, using a Powerwave X340 microplate reader (Bio-TEK Instrument Inc., Winooski, VT). The K_m and V_{max} values of the tyrosinase activity were assessed via Lineweaver–Burk plot, using the various concentrations of L-tyrosine substrate.

Cell cultures

All described protocols approved and monitored by the Kubt Institutional Review Board, in accordance with the Declaration of Helsinki Principles. Melan-a cell (murine Melan-a melanocyte) is originally derived from C57BL/6 J (black, a/a) mice, a kind gift from Professor Dorothy C. Bennett (St George's Hospital Medical School, London, UK). This cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin–penicillin (100 μg ml^{-1} each), and 200 nM

12-*o*-tetradecanoylphorbol-13-acetate at 37 °C in an atmosphere containing 5% CO_2. Human epidermal melanocytes, also referred to as HEMn, were obtained by permission according to the Declaration of Helsinki Principles and supplied by Cascade Biologics Inc. (Portland, OR) and were grown in Medium 154 (Cascade Biologics Inc.) supplemented with human melanocyte growth supplement (Cascade Biologics Inc.), 200 nM 12-*o*-tetradecanoylphorbol-13-acetate, and α-melanocyte-stimulating hormone (1×10^{-6} M) at 37 °C in an atmosphere containing 5% CO_2. Monolayers of confluent melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Gibco BRL).

Cell viability assay

Cell viability was determined via crystal violet staining. After 4 days of incubation with the test substances, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. The cells were then stained for a further 5 minutes at room temperature, and then rinsed three times. The crystal violet retained by the adherent cells was extracted using 95% ethanol, and absorbance was determined at a wavelength of 590 nm.

Inhibitory effect of melanin production in *S. bikiniensis*

A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated into a Papavizas' VDYA agar slant, containing 200 ml of V-8 juice (Campbell Soup Co., Camden, NJ), 2 g of glucose, 2 g of yeast extract (Difco, Sparks, MD), 1 g of $CaCO_3$, 20 g of agar (Difco), and 800 ml of distilled water, adjusting the pH to 7.2. After 2 weeks of incubation at 28 °C, the spore suspension obtained was then transferred to a series of sterile microtubes. A volume of 0.4 ml of the suspension of *S. bikiniensis* spores was then added to agar medium ISP no. 7 (40 ml), which had been supplemented with 0.2% Bacto-yeast extract (Difco), and was spread over the surface of the agar uniformly with a glass hockey bar. After the surface of the agar had dried, a paper disc (8 mm diameter) soaked with the sample solution was positioned on the agar plate. The plate was incubated at 28 °C for 48 hours; the zone of melanin formation was measured as diameter (mm) from the reverse side of the plate (Lee *et al.*, 1997).

Measurement of melanin content

The cells were seeded in a 24-well plate (Falcon) at a density of 1×10^5 cells per well and were allowed to attach overnight. Then the cells were incubated in a fresh medium containing various concentrations of compounds for 4 days. After the cells had been washed with phosphate-buffered saline, they were lysed with 250 μl of 0.85 N KOH and transferred to a 96-well plate. The melanin contents were estimated via the measurement of absorbance at a wavelength of 405 nm (Bennett *et al.*, 1987).

Western blot analysis

Cells were lysed in cold lysis buffer (0.1 M Tris–HCl, pH 7.2, 1% Nonidet P-40, 0.01% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin). A 30 μg portion of proteins was separated via 8% SDS-PAGE and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat skim milk in a Tris-buffered saline-T buffer. Tyrosinase, TRP-1, TRP-2, and β-actin were all detected with a rabbit polyclonal anti-αPEP7 antibody, a rabbit polyclonal anti-αPEP1 antibody, a rabbit polyclonal anti-αPEP8 antibody (a gift from Dr VJ Hearing at National Institutes of Health, Bethesda, MD) and a mouse monoclonal anti-β-actin antibody (Sigma), respectively. Phospho-specific ERK1/2, and both phosphorylated and non-phosphorylated ERK1/2 were acquired from Cell Signaling Technology (Beverly, MA), and microphthalmia Ab-1 was obtained from NeoMarkers (Fremont, CA). The cells were further incubated with horseradish peroxidase-conjugated secondary antibody. All bound antibodies were then detected with an Amersham ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

RT-PCR

Total RNA was isolated from cells using an RNease Mini Kit (Qiagen, Valencia, CA). Then, 1 μg of RNA was reverse transcribed using the ImProm II Reverse Transcription System (Promega, Madison, WI). The cDNA obtained was amplified with the following primers: MITF (366 bp product) forward 5′-TCCGTCTCTCACTGGATTGGTG-3′ and reverse 5′-CGTGAATGTGTGTTCATGCCTGG-3′. The PCR conditions were follows: 30 cycles of 1 minute at 94 °C, 1 minute at 55 °C, and finally 1 minute at 72 °C, and the resulting PCR products were visualized by electrophoretic separation on 1.5% agarose gels with ethidium bromide staining. Specific primers for glyceraldehyde-3-phosphate dehydrogenase were added as a control.

UVB-induced hyperpigmentation in brown guinea-pigs

UVB-induced hyperpigmentation was induced on the backs of the brown guinea-pigs weighing approximately 500 g (SLC, Shizuoka, Japan) using a slight modification of the methods reported by Ando *et al.* (1998) and Imokawa *et al.* (1986). The guinea-pigs were anesthetized with pentobarbital (30 mg kg^{-1}), and separate areas (1 cm diametrical circle) of the back of each animal were exposed to the UVB radiation (Waldmann UV 800, Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280–305 nm). The total UVB dose was 500 mJ cm^{-2} per exposure. Groups of four animals were used in the experiments. The animals were exposed to the UVB radiation once a week for 3 consecutive weeks. The candidate for the whitening agent was given the material topically to the hyperpigmented areas (1% in propyleneglycol/EtOH/H_2O (5:3:2), 5 μl per circle) twice a day for 4 weeks from the next day of the last tanning. The degree of pigmentation was assessed by the *L*-value measured using a chromameter (CR-300, Minolta, Tokyo, Japan). The *ΔL*-value was evaluated according to the *L*-value (brightness index) measured by the chromameter, as follows: $\Delta L = L$ (at each day measured)$-L$ (at day 0). A reduction in the *ΔL*-value indicates UVB-induced skin pigmentation.

Origin and maintenance of parental fish

Adult zebrafishes were obtained from a commercial dealer and 10–15 fishes were kept in 5 l acrylic tank with the following conditions: 28.5 °C, with a 14/10 hours light/dark cycle. Zebrafishes were fed three times a day, 6 days per week, with TetraMin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 minutes (Choi *et al.*, 2007).

Compounds treatment and phenotype-based evaluation and tyrosinase activity

Synchronized embryos were collected and arrayed by pipette (3–4 embryos per well in 96-well plates containing 200 μl of embryo medium). Test compounds were dissolved in 0.1% DMSO, and then

added to the embryo medium from 9 to 72 hours post-fertilization (h.p.f.) (63 hours exposure). The effects on the pigmentation of zebrafish were observed under the stereomicroscope. Occasional stirring as well as replacement of the medium was performed daily to ensure the even distribution of the compounds. In all experiments, 0.2 mM PTU was used to generate transparent zebrafish without interfering in the developmental process (Karlsson *et al*., 2001), and considered as a standard positive control. Phenotype-based evaluations of body pigmentation were carried out at 28 and 58 h.p.f. For observation, embryos were dechorionated by forceps, anesthetized in tricaine methanesulfonate solution (Sigma), mounted in 3% methylcellulose on a depression slide (Aquatic Eco-Systems, Apopka, FL), and photographed under the stereomicroscope MZ16 (Leica Microsystems, Ernst-Leitz-Strasse, Germany). Tyrosinase activity was spectrometrically determined as described previously (Busca *et al*., 1996), with minor modification. Briefly, about 100 zebrafish embryos were treated with melanogenic modulators from 9 to 48 h.p.f. and sonicated in Pro-Prep protein extraction solution (Intron, Daejeon, Korea). The lysate was clarified by centrifuging at 10,000 *g* for 5 minutes. After quantification, 250 μg of total protein was added followed by 100 μl of 1 mM L-DOPA. Control well contained 100 μl of lysis buffer and 100 μl of 1 mM L-DOPA. After incubation for 60 minutes at 37 °C, absorbance was measured at 475 nm using the SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). All experiments were performed at least three times with similar results (Choi *et al*., 2007).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by a grant (PDM1600526) from Plant Diversity Research Center of the 21st Century Frontier Research Program, which is funded by the Ministry of Science and Technology of the Korean Government and supported by a grant (code no. 2007030134039) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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