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Inhibitory effect of *p*-coumaric acid by *Rhodiola sachalinensis* on melanin synthesis in B16F10 cells

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Rhodiola has been widely used in traditional Asian medicine. In this study, we tested the hypopigmentation effects of *R. sachalinensis* and its active compounds including catechin, chlorogenic acid, *p*-coumaric acid, and *p*-tyrosol. Results have shown that only *p*-coumaric acid inhibits melanin synthesis in B16F10 cells. However, *p*-coumaric acid did not inhibit tyrosinase activity when L-DOPA was used as a substrate. Instead, *p*-coumaric acid inhibited tyrosinase activity when L-tyrosine was used as a substrate. We further analyzed the changes of cAMP responsive element binding protein (CREB) phosphorylation and tyrosinase gene expression. The results indicate that *p*-coumaric acid does not affect CREB phosphorylation or tyrosinase protein production. In turn, these findings demonstrate that *p*-coumaric acid has no effect on the upstream regulation of tyrosinase gene expression, although *p*-coumaric acid showed a significant inhibitory effect on melanogenesis. Because *p*-coumaric acid showed different effects on tyrosinase activity according to different substrates, we tested whether tyrosinase can utilize *p*-coumaric acid as a substrate. Our findings revealed that competitive inhibition occurs between *p*-coumaric acid and tyrosine. Consequently, this finding could be a primary mechanism for the hypopigmenting action of *p*-coumaric acid.

1. Introduction

Rhodiola is a genus of alpine plant containing over 200 species which grow throughout Europe, Asia, and Alaska. *Rhodiola* has been widely used in traditional Asian medicine as a means to stimulate the nervous system, decrease depression and fatigue, in addition to help in preventing high altitude sickness. Past studies have also reported that *Rhodiola* has diverse biological functions and strong antioxidant activities. In this study, we tested the hypopigmenting effects of *R. sachalinensis* and sought the ingredients of hypopigmenting activity.

R. sachalinensis is known to have diverse active compounds including salidroside, flavonoids (catechin including EGCG, Mook-Jung et al., 2002) rhodioflavonoside, organic acids (gallic acid, chlorogenic acid, Lee et al., 2000; Ohsugi et al., 1999) *p*-coumaric acid (trans-*p*-hydroxycinnamic acid), and *p*-tyrosol (Ming et al., 2005).

In this study, we observed the hypopigmenting effects by *R. sachalinensis* using B16F10 cells. Among these active compounds, EGCG is reported to inhibit melanin synthesis via decreased MITF production (Kim et al., 2004). In addition, gallic acid also inhibits melanogenesis by inhibiting tyrosinase activity (Kim, 2007). In order to discover additional ingredients with hypopigmenting effects in *R. sachalinensis*, we tested catechin, chlorogenic acid, *p*-coumaric acid, and *p*-tyrosol. We found that among these compounds, only *p*-coumaric acid inhibits melanin synthesis in B16F10 cells. Furthermore, we analyzed the hypopigmenting mechanism of *p*-coumaric acid.

Abbreviations: α-MSH, α-melanocyte stimulating hormone; CREB, cAMP responsive element binding protein; L-DOPA, dihydroxyphenylalanine; MITF, microphthalmia-associated transcription factor

2. Investigations and results

2.1. Effect of R. sachalinensis extract on α-MSH induced melanogenesis

B16F10 cells were treated with *R. sachalinensis* extracts (50 μg/ml and 100 μg/ml) in the presence of 1 μM of α-MSH. *R. sachalinensis* extracts were not cytotoxic (data not shown) and showed inhibitory effects on melanogenesis in B16F10 cells (Fig. 1).

2.2. Effects of p-tyrosol, chlorogenic acid, catechin, and p-coumaric acid on melanin synthesis in B16 F10 cells

p-Tyrosol, chlorogenic acid, catechin, and *p*-coumaric acid are known as the primary ingredients of *R. sachalinensis*. To investigate the effects of these substances, B16F10

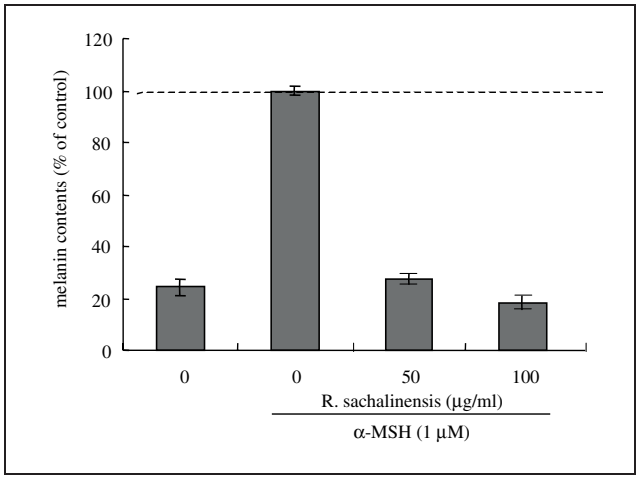

Fig. 1: Inhibitory effect of *Rhodiola sachalinensis* on melanogenesis in B16F10 cells. B16F10 cells were treated with *R. sachalinensis* extracts (50 μg/ml or 100 μg/ml) in the presence of α-MSH (1 μM). Each determination was made in triplicate and the values are expressed as means ±SD

cells were co-treated with α-MSH (1 μM) at increasing doses (1–50 μM) of *p*-tyrosol, chlorogenic acid, catechin, and *p*-coumaric acid (Fig. 2). Among these substances, only *p*-coumaric acid showed a dose-dependent inhibitory effect on melanogenesis (Fig. 2D). The other substances showed no inhibitory effects (Fig. 2A, B, C). As shown in Fig. 2D, the melanin content was significantly down-regulated and inhibited by 36% and 60% at 10 μM and 50 μM of *p*-coumaric acid, respectively. Furthermore, *p*-coumaric acid did not show any effect on cell viability at 0.1–50 μM (Fig. 3).

2.3. Effect of p-coumaric acid on tyrosinase activity

To further investigate the mechanism of *p*-coumaric acid on tyrosinase activity, we tested whether *p*-coumaric acid can inhibit tyrosinase activity. To achieve this, B16F10 cells were co-treated with α-MSH and *p*-coumaric acid. We found *p*-coumaric acid not to have any inhibitory effect on tyosinase activity, when L-DOPA oxidation method was used in the mixture (Fig. 4A). Next, we examined if *p*-coumaric acid can affect L-DOPA oxidation in a cell free system and found that *p*-coumaric acid did not show any inhibitory effect (Fig. 4B).

2.4. Effect of p-coumaric acid on phosphorylation CREB and tyrosinase expression

To further confirm the effects of *p*-coumaric acid on tyrosinase gene expression, we studied whether *p*-coumaric acid can affect CREB phosphorylation, which is known to be involved in tyrosinase gene expression. As shown in Fig. 5, *p*-coumaric acid did not show any effect on α-MSH-induced increased level of *p*-CREB and tyrosinase protein.

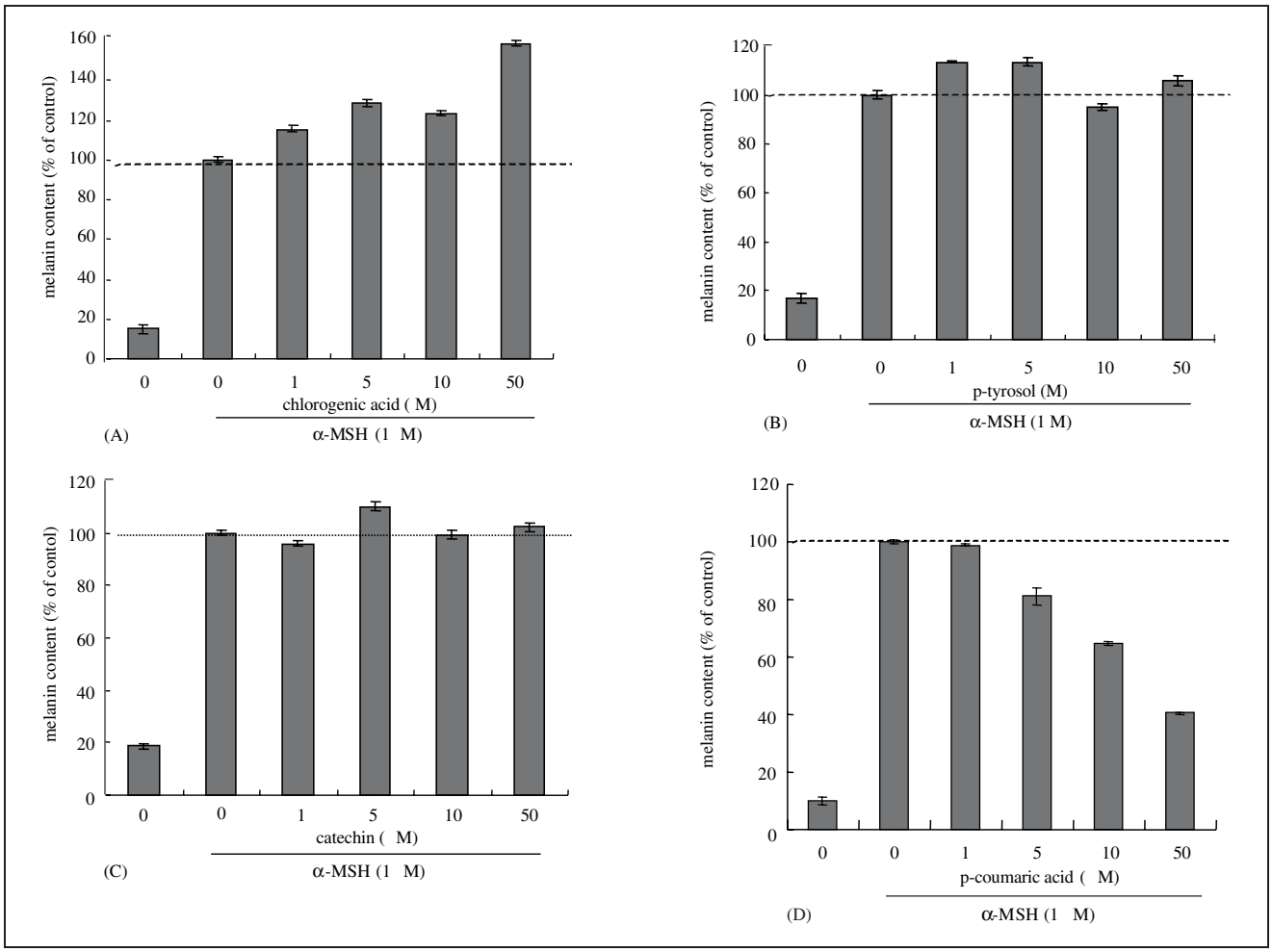

Fig. 2: Inhibition of α-MSH–induced melanogenesis by *p*-coumaric acid. B16F10 cells were treated with chlorogenic acid (A), *p*-tyrosol (B), catechin (C), and *p*-coumaric acid (D) for 72 h in the presence of α-MSH (1 μM). Melanin content was measured, as described in "Experimental". Values are expressed as means ±SD from three independent experiments

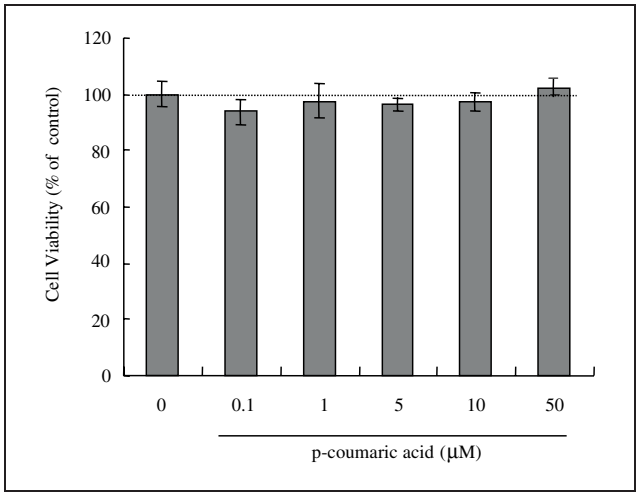

Fig. 3: Effect of *p*-coumaric acid on B16F10 cell viability. B16F10 cells were treated with various concentrations (0.1–50 μM) of *p*-coumaric acid for 24 h in serum-free media. The cell viabilities were determined by the crystal violet assay. Each determination was made in triplicate and the values are expressed as means ±SD

2.5. Competitive inhibition of p-coumaric acid and tyrosine

Our results indicate that *p*-coumaric acid did not inhibit tyrosinase activity or suppress tyrosinase synthesis, although it showed a significant hypopigmenting effect in B16F10 cells. We paid particular attention that *p*-coumaric acid has a similar structure to tyrosine (Fig. 6). Next, we hypothesized that the hypopigmenting effects of *p*-coumaric acid occurred through competitive inhibition between *p*-coumaric acid and tyrosine. To test this hypothesis, we used tyrosine as a substrate instead of L-DOPA. We found that *p*-coumaric acid inhibited tyrosinase activity in a dose-dependent manner, corresponding to ∼33% inhibition at 50 μM and ∼40% inhibition at 100 μM (Fig. 7A). Furthermore, *p*-coumaric acid also inhibited α-MSH-induced tyrosinase activity in the cell system in a dose-dependent manner (Fig. 7B). These results suggest that *p*-coumaric acid inhibits melanin production via competitive inhibition between *p*-coumaric acid and tyrosine. To further investigate whether tyrosinase uses *p*-coumaric acid as a substrate and subsequently generates its corre-

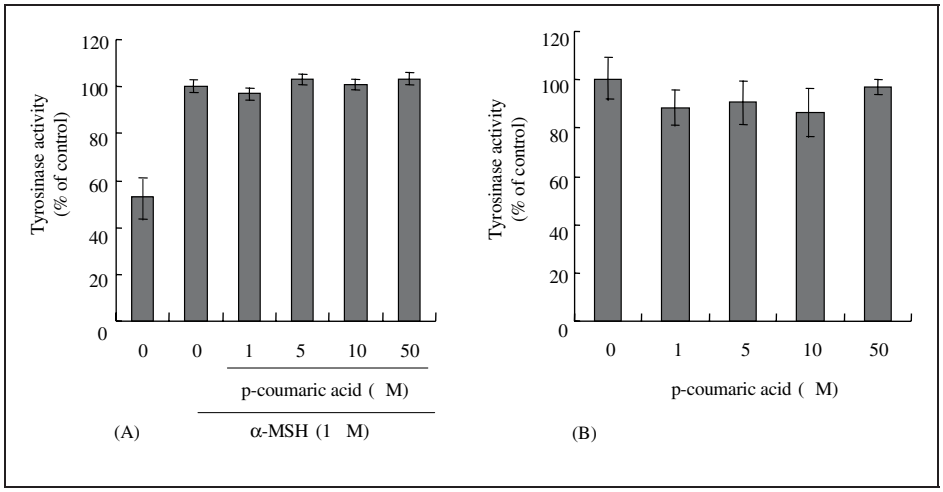

Fig. 4:
Effect of *p*-coumaric acid on tyrosinase activity using L-DOPA as a substrate. Cells were treated with increasing doses of *p*-coumaric acid (1–50 μM) for 72 h in the presence of α-MSH (1 μM). Tyrosinase activity was measured as described in "Experimental" (A). Similarly, tyrosinase activity in a cell-free system (B). Values are expressed as means ±SD from three independent experiment

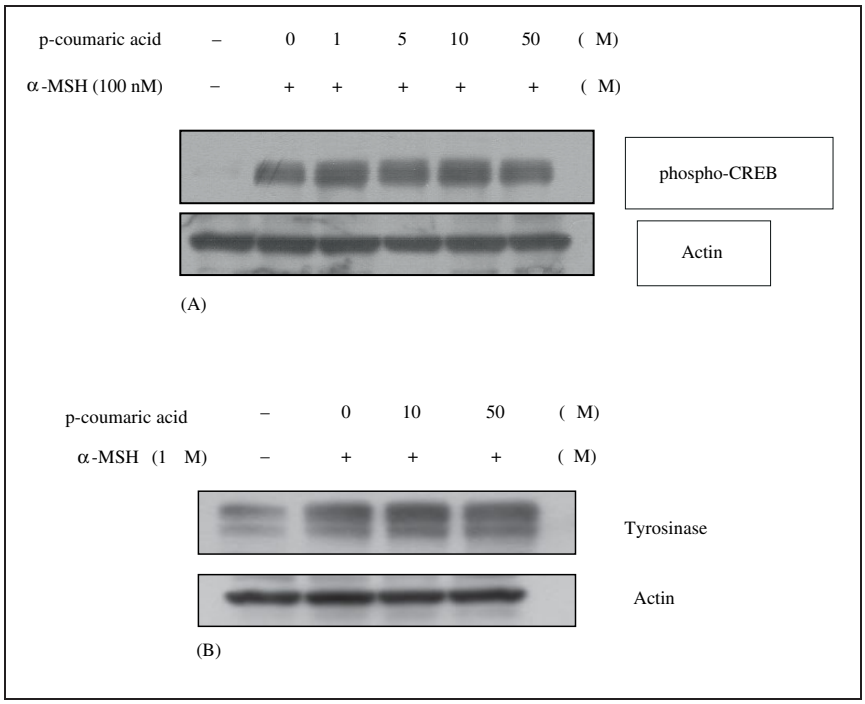

Fig. 5:
Effects of *p*-coumaric acid on α–MSH induced CREB phosphorylation and tyrosinase expression. Cells were treated with *p*-coumaric acid (1, 5, 10, 50 μM) for 3 h in serum-free media, in the presence of α-MSH (1 μM), B16F10 (A). The cells were also treated with 10 and 50 μM of *p*-coumaric acid for 48 h (B). Cell lysates were subjected to Western blot analysis with antibodies against phospho-specific CREB (*p*-CREB) and tyrosinase. The actin antibody was used as a control

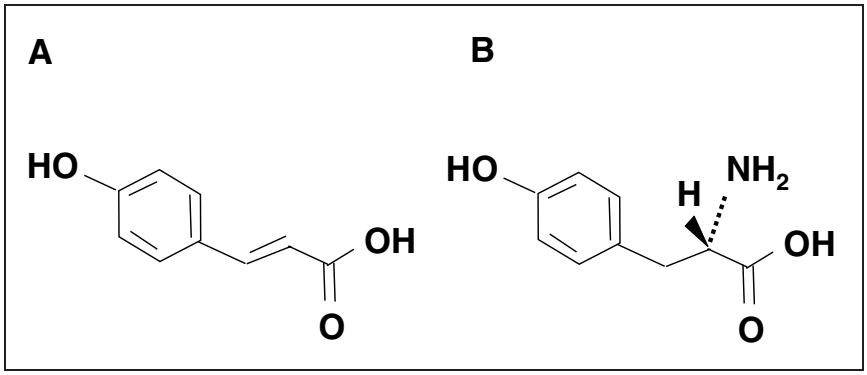

Fig. 6:
Structures of *p*-coumaric acid (A) and L-tyrosine (B)

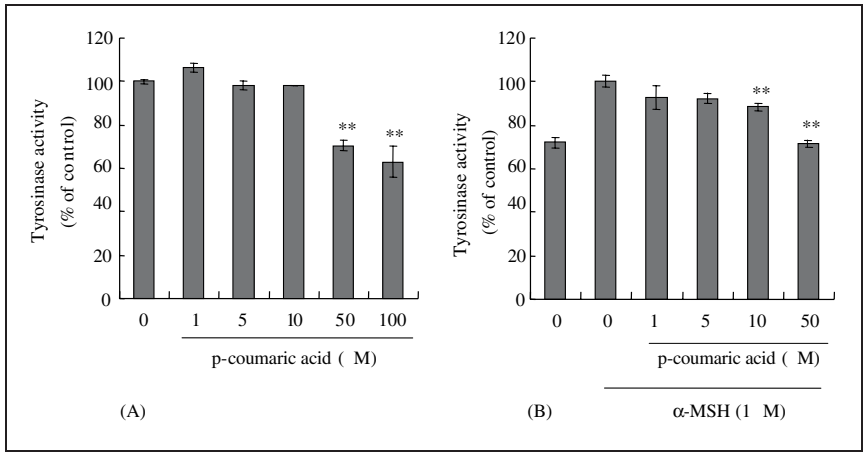

Fig. 7:
Inhibitory effect of *p*-coumaric acid using L-tyrosine as a substrate on tyrosinase activity. L-tyrosine was used as a substrate to test the inhibitory effect of *p*-coumaric acid on tyrosinase activity. The tyrosinase activity was measured in a cell-free system (A) and cell system (B). Values are expressed as means ±SD from three independent experiments. $^{**}p < 0.01$ in comparison to the untreated control

sponding product, we observed changes of UV-Vis absorption spectrum peaks. We found that the peak shifted from 286 nm to 246 nm when tyrosinase metabolizes *p*-coumaric acid (Fig. 8C). This indicates that *p*-coumaric acid inhibits melanogenesis in B16F10 cells via competitive inhibition with tyrosine.

3. Discussion

In this study, *R. sachalinensis* showed a significant inhibitory effect on melanogenesis. Moreover, *p*-coumaric acid (a principal component of *R. sachalinensis*) was found to inhibit melanin production in α-MSH-stimulated B16F10 cells (Fig. 2D). However, the molecular mechanism by which *p*-coumaric acid inhibits melanin synthesis in B16F10 cells remains to be identified. In mammals, melanogenesis is regulated by three melanocyte specific enzymes: tyrosinase, tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2). Tyrosinase is a rate-limiting key enzyme in the pathway of melanogenesis and catalyzes the hydroxylation of tyrosine to 3,4-dihydoxyphenylalanine (DOPA) as well as the oxidation of DOPA to dopaquinone (Hearing and Jimenez 1989). Thus, the regulation of tyrosinase plays an important role in melanogensis (Hearing and Tsukamoto 1991). We found that *p*-coumaric acid did not inhibit tyrosinase activity

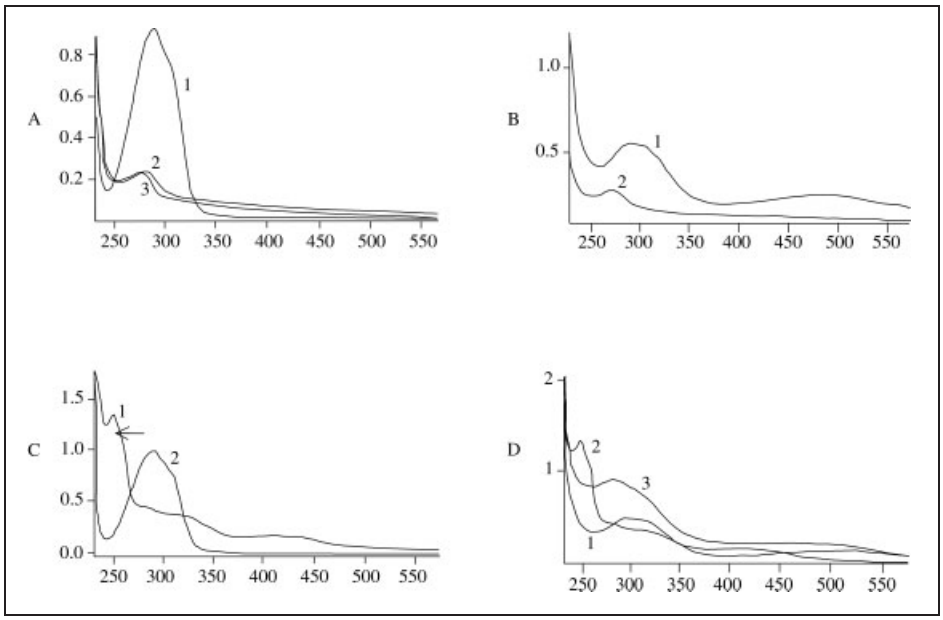

Fig. 8:
Competitive inhibition of *p*-coumaric acid and tyrosine. Absorption spectrums obtained from (A) *p*-coumaric acid 50 μM (1), tyrosine 50 μM (2), tyrosinase (3), (B) tyrosine 50 μM and tyrosinase (1), tyrosine 50 μM (2), (C) *p*-coumaric acid 50 μM and tyrosinase (1), *p*-coumaric acid 50 μM (2), (D) tyrosine 50 μM and tyrosinase (1), *p*-coumaric acid 50 μM and tyrosinase (2), *p*-coumaric acid 50 μM, tyrosine 50 μM, and tyrosinase (3). All reactions were maintained at 37 °C for 10 min

when L-DOPA was used as a substrate. Conversely, *p*-coumaric acid inhibited tyrosinase activity when L-tyrosine was used as a substrate. In order to analyze the hypopigmenting effects of *p*-coumaric acid, the transcription regulation of the tyrosinase gene was investigated. Cyclic AMP has been reported to be a key messenger in the regulation of skin pigmentation (Busca and Ballotti 2000). In addition, α-MSH (α-melanocyte stimulating hormone) can increase intracellular cAMP content by the activation of adenylate cyclase (Mas et al. 2003). As a result, cAMP can activate PKA, which in turn, phosphorylates CREB (cAMP responsive element binding protein – known as a transcription factor) (Sassone-Corsi 1998). Once phosphorylated, CREB can up-regulate MITF (microphthalmia-associated transcription factor), which binds to the M-box and E-box motifs in the tyrosinase gene promoter for the transcriptional regulation of the tyrosinase gene (Bertolotto et al. 1998; Ganss et al., 1994). Western blot analysis showed that *p*-coumaric acid does not affect CREB phosphorylation or tyrosinase protein production (Fig. 4). These results suggest that *p*-coumaric acid has no effect on the upstream regulation of tyrosinase gene expression, although *p*-coumaric acid showed a significant inhibitory effect on melanogenesis (Fig. 5 A, B).

As already described, *p*-coumaric acid shows different actions depending on the assay method used to investigate different substrates. Because *p*-coumaric acid is similar in structure with tyrosine, we hypothesized that the effects by *p*-coumaric acid occurred through competitive inhibition between *p*-coumaric acid and tyrosine. As expected, the absorption spectrum analysis demonstrated that tyrosinase utilized *p*-coumaric acid as a substrate and produced another product which was shown by the changes in the absorption spectrum (Fig. 8). These findings clearly explain the mechanism by which *p*-coumaric acid inhibits melanogenesis in B16F10 cells.

In conclusion, we report here that the hypopigmenting effect by *p*-coumaric acid occurs through competitive inhibition between *p*-coumaric acid and tyrosine. In addition, cytotoxicity was not observed up to 1 mM concentration (data not shown). Hence, *p*-coumaric acid can be may contribute used safely to make hypopigmenting agents. In addition, *p*-coumaric acid can to the hypopigmenting effect, if it is used with another hypopigmenting agent which acts with a different mechanism.

4. Experimental

4.1. Materials

We obtained *p*-coumaric acid, L-DOPA, mushroom tyrosinase, tyrosine and α-melanocyte-stimulating hormone (α-MSH) from Sigma (St. Louis, MO, USA). In addition, an antibody recognizing phospho-CREB (Ser 133, #1996) was purchased from Cell Signaling Technology (Beverly, MA, USA); tyrosinase (C-19) and actin (I-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture

B16F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml of penicillin at 37 °C in a 5% CO_2 atmosphere.

4.3. Preparation of Rhodiola sachalinensis extracts

R. sachalinensis was thoroughly dried and ground into a powder, and 50% EtOH was added to extract soluble components at room temperature. Next, the extracts were filtered through Whatman filter paper (Whatman Ltd, Maidstone, England) and subsequently concentrated.

4.4. Cell viability assay

Cell viability was determined using a crystal violet assay (Dooley et al. 1994). After incubating with the test substance for 24 h, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. Next, the cells were stained for 5 min at room temperature and rinsed four times with distilled water. The crystal violet retained by the adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader.

4.5. Measurement of melanin content

B16F10 cells were seeded in 6 well plates at a density of 1×10^5 cells/well for 24 h to allow for attachment. Next, the cells were treated with test substance and incubated for 3 days in the presence of α-MSH (1 μM). The supernatant was taken and the amount of melanin was measured at 400 nm. Following this, the cells were enumerated using a hemocytometer.

4.6. Measurement of tyrosinase activity

B16 F10 cells were treated with *p*-coumaric acid in the presence of α-MSH for 72 h. Next, the cells were washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. After freezing and thawing, the lysates were clarified by centrifugation at 10,000 *g* for 5 min. After adjusting the protein concentrations, 90 μl of lysates containing the same amount of protein, were placed in each well of a 96-well plate, and 10 μl of 10 mM L-DOPA or 500 μM L-tyrosine was added. For the blank, 90 μl of lysis buffer and 10 μl of 10 mM L-DOPA or 500 μM L-tyrosine was added. After incubation at 37 °C, absorbance was measured every 10 min for at least 1 h at 475 nm using an ELISA reader.

4.7. Measurement of tyrosinase activity in a cell-free condition

A cell-free assay system was used to test for direct effects on tyrosinase activity. Seventy microliters of phosphate buffer containing *p*-coumaric acid were mixed with 20 μl of 50 μg/ml mushroom tyrosinase. Next, 10 μl of 10 mM L-DOPA or 500 μM of L-tyrosine were added. Following incubation at 37 °C, absorbance was measured at 475 nm.

4.8. Western blot analysis

B16F10 cells were co-treated with *p*-coumaric acid and α-MSH for 3 h to detect *p*-CREB, or for 48 h to detect tyrosinase. The cells were lysed in cell lysis buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitor (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA]. The blots were incubated with the primary antibodies at a dilution of 1 : 1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

4.9. Competitive inhibition

Phosphate buffer (pH 6.8) with mushroom tyrosinase (100 μg/ml) was mixed with L-tyrosine (50 μM) or *p*-coumaric acid (50 μM) in phosphate buffer to make a 3 ml mixture. Following incubation at 37°, absorbance was scanned (220–500 nm). The absorbance was recorded using a Perkin Elmer spectrophotometer (MBA 2000, Waltham, MA, USA).

4.10. Statistics

Statistical comparison of the data was achieved using the Student's t-test. Statistical significance was set a priori at $p < 0.01$.

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References

Bertolotto C, Busca R, Abbe P, Bille K, Aberdam E, Ortonne JP, et al. (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. Mol Cell Biol 18: 694–702.

Busca R, Ballotti R (2000) Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res 13: 60–69.

Dooley TP, Gadwood RC, Kilgore K, Thomasco LM (1994) Development of an in vitro primary screen for skin depigmentation and antimelanoma agents. Skin Pharmacol 7: 188–200.

Ganss R, Schutz G, Beermann F (1994) The mouse tyrosinase gene. Promoter modulation by positive and negative regulatory elements. J Biol Chem 269: 29808–29816.

Hearing VJ, Jimenez M (1989) Analysis of mammalian pigmentation at the molecular level. Pigment Cell Res 2: 75–85.

Hearing VJ, Tsukamoto K (1991) Enzymatic control of pigmentation in mammals. Faseb J 5: 2902–2909.

Kim DS, Park SH, Kwon SB, Li K, Youn SW, Park KC (2004) (-)-Epigallocatechin-3-gallate and hinokitiol reduce melanin synthesis via decreased MITF production. Arch Pharm Res 27: 334–339.

Kim YJ (2007) Antimelanogenic and antioxidant properties of gallic acid. Biol Pharm Bull 30: 1052–1055.

Lee MW, Lee YA, Park HM, Toh SH, Lee EJ, Jang HD, et al. (2000) Antioxidative phenolic compounds from the roots of Rhodiola sachalinensis A. Bor. Arch Pharm Res 23: 455–458.

Mas JS, Gerritsen I, Hahmann C, Jimenez-Cervantes C, Garcia-Borron JC (2003) Rate limiting factors in melanocortin 1 receptor signalling through the cAMP pathway. Pigment Cell Res 16: 540–547.

Ming DS, Hillhouse BJ, Guns ES, Eberding A, Xie S, Vimalanathan S, et al. (2005) Bioactive compounds from Rhodiola rosea (Crassulaceae). Phytother Res 19: 740–743.

Mook-Jung I, Kim H, Fan W, Tezuka Y, Kadota S, Nishijo H, et al. (2002) Neuroprotective effects of constituents of the oriental crude drugs, Rhodiola sacra, R. sachalinensis and Tokaku-joki-to, against beta-amyloid toxicity, oxidative stress and apoptosis. Biol Pharm Bull 25: 1101–1104.

Ohsugi M, Fan W, Hase K, Xiong Q, Tezuka Y, Komatsu K, et al. (1999) Active-oxygen scavenging activity of traditional nourishing-tonic herbal medicines and active constituents of Rhodiola sacra. J Ethnopharmacol 67: 111–119.

Sassone-Corsi P (1998) Coupling gene expression to cAMP signalling: role of CREB and CREM. Int J Biochem Cell Biol 30: 27–38.