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Resveratrol as a k_{cat} type inhibitor for tyrosinase: Potentiated melanogenesis inhibitor

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ABSTRACT

Resveratrol exhibited the inhibitory activity against mushroom tyrosinase (EC1.14.18.1) through a k_{cat} inhibition. Resveratrol itself did not inhibit tyrosinase but rather was oxidized by tyrosinase. In the enzymatic assays, resveratrol did not inhibit the diphenolase activity of tyrosinase when L-3,4-dihydroxyphenylalanin (L-DOPA) was used as a substrate; however, L-tyrosine oxidation by tyrosinase was suppressed in presence of 100 μM resveratrol. Oxidation of resveratrol and inhibition of L-tyrosine oxidation suggested the inhibitory effects of metabolites of resveratrol on tyrosinase. After the 30 min of preincubation of tyrosinase and resveratrol, both monophenolase and diphenolase activities of tyrosinase were significantly suppressed. This preincubational effect was reduced with the addition of L-cysteine, which indicated k_{cat} inhibition or suicide inhibition of resveratrol. Furthermore, investigation was extended to the cellular experiments by using B16-F10 murine melanoma cells. Cellular melanin production was significantly suppressed by resveratrol without any cytotoxicity up to 200 μM. *trans*-Pinosylvin, *cis*-pinosylvin, dihydropinosylvin were also tested for a comparison. These results suggest that possible usage of resveratrol as a tyrosinase inhibitor and a melanogenesis inhibitor.

1. Introduction

Melanogenesis is the process of melanin production by melanocytes within the skin and hair follicles and is mediated by several enzymes such as tyrosinase, TRP-1 and TRP-2.[1] Tyrosinase is the key enzyme in melanin synthesis. This copper-contained multi-functional oxidase catalyzes hydroxylation of L-tyrosine to L-DOPA and further oxidation of L-DOPA to dopaquinone.[2] In various living systems, melanin is responsible for pigmentation and other functions such as chelation; hence, alterations in melanin synthesis occur in many disease states such as melanoma or Parkinson's disease. Melanoma is one of the most commonly diagnosed diseases,[3] and its incidence is raising a world-wide concern.[4] Melanoma-specific anticarcinogenic activity is known to be linked with tyrosinase activity.[5] Furthermore, melanin production-mediated browning is a major concern in food industries and cosmetic companies. Thus, development of non-toxic antibrowning reagents is essential. Previously, the most widely spread method for antibrowning was the use of sulfiting agents,[6] but Food and Drug Administration has banned sulfate agent for fruits and vegetables.[7] Hence, immediate finding of a replacement of antibrowning reagents is essential. As the purpose of applications, naturally occurring substances are usually more favorable than synthetic ones. The most common natural antibrowning agent is ascorbic acid. However, the effect of ascorbic acid against enzymatic oxidation is temporary since it is chemically oxidized to non-functional form, dehydroascorbic acid.[8] These problems prompt us to search safer and more effective melanin formation inhibitors from natural sources.

Resveratrol, 3,5,4′-trihydroxy-*trans*-stilbene (**1**; see Fig. 1 for the structure), is a widely distributed natural stilbenoid in nature such as in grapes. Resveratrol is currently the subject of many research investigations due to its health beneficial effects including antioxidative, antifungal, anticarcinogenic, cardioprotective, and anti-aging actions.[9–12] Antioxidant effect of resveratrol has been extensively studied, and the mechanism of action involves the recruitment of antioxidant defense enzymes in the cells.[13–17] In addition to antioxidant effect, resveratrol is known to have anti-cancerous and apoptotic actions.[10,18,19] Despite of wide beneficial biological functions, its effects on melanogenesis and on tyrosinase have been limitedly studied. Inhibitory effect of resveratrol on tyrosinase activity have been reported previously.[20,21] Newton et al., also previously reported that antimelanogenic effect on human melanocyte through affecting the post-transcriptional synthesis of tyrosinase.[22] However, the detailed mechanism of tyrosinase inhibition is not fully understood. Furthermore, the effects of resveratrol on monophenol oxidation of tyrosinase and on cellular melanogenesis as a potentiated drug have not been studied yet.

k_{cat} Type inhibitors possess reactive groups selectively activated by target enzyme at its active site.[23] They are also often described

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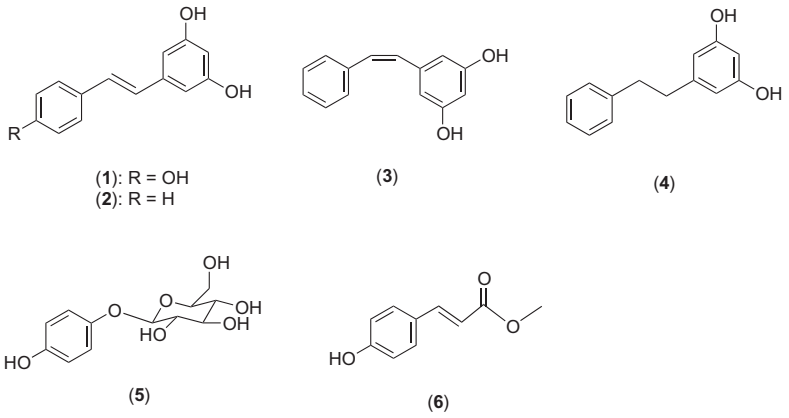


Figure 1. Structure of resveratrol and the related compounds.

as a suicide inhibitors. The enzyme sometimes undergoes inactivating processes by bioactivating their substrates; in the case of tyrosinase, the reactive oxidation product(s) was converted from unreactive tyrosinase substrate to inactivate enzyme.[24,25] Several compounds including natural products are known to be a suicide inhibitor; for example, 7,8,4′-trihydroxyisoflavone and 5,7,8,4′-tetrahydroxyflavone are recently reported as one of the potent suicide inhibitors of tyrosinase.[26] The investigation of k_{cat} type inhibitors is important in functional design of enzymatic inhibitors for pharmaceutical and therapeutic purposes. In the case of tyrosinase, functional inhibitors are not used only in pharmaceutical field but also used in food industry and for cosmetic users. These problems motivate us to find the inhibitory mechanism of resveratrol on tyrosinase activity and on melanogenesis.

2. Materials and methods

2.1. Materials

Resveratrol (**1**) and arbutin (**5**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine, L-cysteine, L-DOPA, BHA and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-L-tyrosine was purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). *trans*-Pinosylvin (**2**), *cis*-pinosylvin (**3**), dihydropinosylvin (**4**), methyl *p*-coumarate (**6**) were synthesized and obtained from the previous studies.

2.2. Enzyme/spectrophotometric assay

General procedures were the same as the previous work[27,28] but slightly modified. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. and was purified by anion-exchange chromatography using DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) as previously described.[29] The current experiment was subjected to use the purified tyrosinase. Although mushroom tyrosinase differs somewhat from those of other sources, this fungal enzyme was used for the entire experiment because it is readily available. Throughout the experiment, L-DOPA or L-tyrosine was used as a substrate. In a spectrophotometric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a SpectraMAX Plus Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 30 °C. All samples were first dissolved in DMSO and used for the experiment after dilution. The final concentration of DMSO in the test solution was always 3.3%. The assay was performed as previously reported with slight modifications. First, 100 μL of a 3 mM L-DOPA or L-tyrosine aqueous solution was mixed with 2.1 mL of filtered distilled H_2O and 600 μL of 67 mM phosphate buffer (pH 6.8) and incubated at 30 °C for 5 min. Then, 100 μL of the sample solution and 100 μL of the same phosphate buffer solution of the purified mushroom tyrosinase (1 μg/mL) were added in this order to the mixture. The assays were performed in triplicate on separate occasions. Arbutin was also tested as a comparison.

2.3. Oxygen consumption assay

In general, procedure was previously described.[27,28] Briefly, 100 μL of a 3 mM L-DOPA or L-tyrosine aqueous solution was mixed with 2.1 mL of distilled H_2O, 600 μL of 67 mM phosphate buffer (pH 6.8) and 100 μL of sample-DMSO solution was incubated at 30 °C for 5 min. Then, 100 μL of the same phosphate buffer solution of the purified mushroom tyrosinase (1 μg/mL) was added and oxygen consumption was measured with an OBH 100 oxygen electrode and an oxygraph equipped with a water-jacket chamber of YSI 5300 (all from Yellow Springs Instruments Co., Yellow Springs, OH) maintained at 30 °C for 60 min. The results were expressed as the oxygen consumption in μM, and calibration of an oxygen electrode was performed by using 4-*tert*-butylcatechol and excess tyrosinase according to the previous report.[30] All assays were performed in triplicate on separate occasions.

2.4. HPLC analysis

Time-dependent consumption of substrates and/or formation of products were monitored with HPLC analysis. The HPLC analysis was performed on an EYELA LPG-100 (Tokyo Rikakikai Co. Ltd, Tokyo, Japan) with an EYELA UV-7000 detector (Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and Develosil ODS-UG-5 column (4.6 × 150 mm, Nomura Chemical Co., Ltd, Japan). In general, the operating conditions were as follows: solvent; 7% MeCN/H_2O containing 0.2% TFA, flow rate; 1.0 mL/min, detection; UV at 280 nm, injected amount; 20 μL from above described 3 mL assay system. For analysis, samples were collected from the reaction mixtures described above at certain time points. The peak heights of each chromatographic peak were used to monitor the consumption of substrates and/or formation of products. In appropriate occasions, the results were expressed with the ratio of the height of sample peaks to that of control one, and then points were connected smoothly using the statistical software.

2.5. Cell culture

B16-F10 mouse melanoma cells (CRL-6475) were obtained from ATCC (Manassas, VA, USA), and cultured in continuous log phase

growth in DMEM containing 10% FBS. Cells were seeded in 96-well plates (2000 cells/well) and incubated at 37 °C in 5% CO_2 for about 24 h before chemical treatment. Each chemical was applied in duplicate with a final content of 0.1% DMSO, and treated cells were cultured for 72 h before assays.

2.6. Melanin assay

The melanin content was determined as previously described[31,32] with minor modifications. Cells were washed with PBS, harvested by trypsinization, and centrifuged for 10 min at 1500×*g*. The cell pellets were then dissolved in 1.0 M NaOH containing 10% DMSO during 2 h incubation at 80 °C. Melanin content was measured at 475 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices, Union City, CA, USA).

2.7. Cell viability assays

Cell viability was determined by trypan blue exclusion and MTT cell proliferation assays. Both bioassays basically provided the same results but the concentration leading to 50% viable cells lost (IC_{50}) was established by trypan blue assay for steady comparison purpose. The appropriate concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay using a Nikon Diaphoto TMD (Nikon, Tokyo, Japan).

2.8. Trypan blue method

Cells were washed with PBS, and dispersed by trypsinization. An aliquot of the cells was mixed with a half volume of DMEM containing 10% FBS, and then mixed with trypan blue solution (final content 0.1%) at room temperature. Unstained cells (viable cells) were counted using a hemocytometer within 10 min after mixing with trypan blue solution.

2.9. MTT method

Cells were washed with PBS, and dispersed with trypsinization, and an aliquot of the cells was seeded in 96-well plates and incubated with DMEM containing 10% FBS at 37 °C in 5% CO_2 for 16–24 h. At the end of the period, 10 μl of MTT reagent were added to each well, which was then incubated at 37 °C in 5% CO_2 for 4 h. Then, 100 μl of detergent reagent were added to each well. The plate was kept at room temperature in the dark for 2 h, and a relative amount of MTT reduction was determined based on the absorbance at 570 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices).

2.10. Statistical analysis

The statistical significance of differences was evaluated by either Student's or Welch's *t*-test after examining the variances using F-test and $^{**}p$ <0.01 was considered to be statistically significant.

3. Results

3.1. Oxidation of resveratrol by mushroom tyrosinase

The investigation was begun with finding whether tyrosinase oxidized resveratrol or not since the previous data suggested that *para*-4-hydroxyl unit with no steric hindrance was often oxidized by tyrosinase. Resveratrol (100 μM) was incubated with tyrosinase for 60 min, and its conversion at each time point was monitored with reverse-phase HPLC system. Resveratrol (peak ***a***; t_R = 9.0 min) was oxidized by tyrosinase in a time-dependent manner (Fig. 2). Most of the oxidation of resveratrol was occurred for the first 30 min, and, at the end of the reaction period, about 90% of resveratrol (peak high ratio) was oxidized. Various tyrosinase-catalyzed

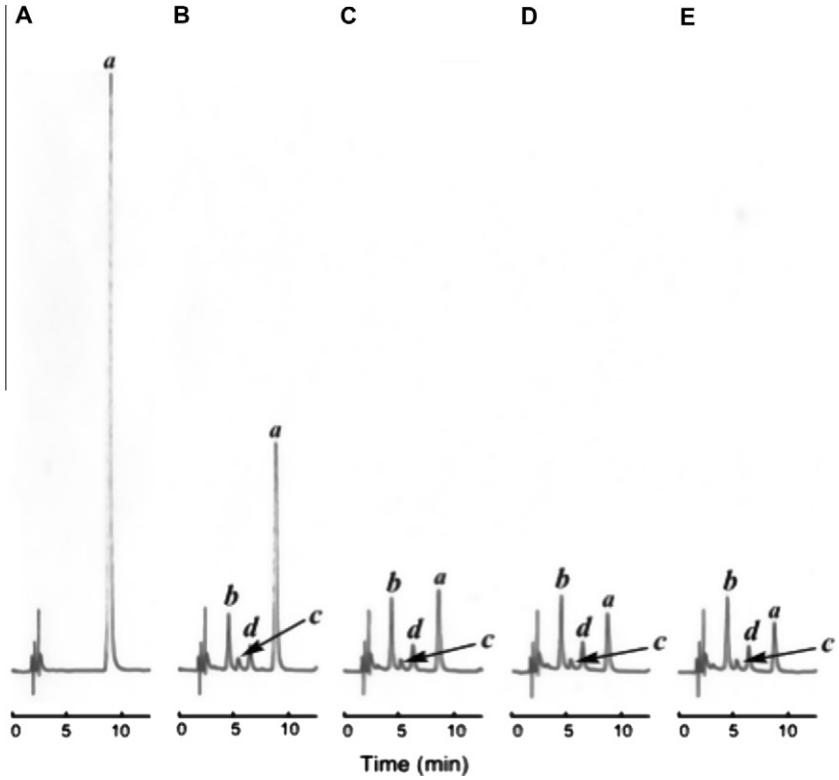


Figure 2. HPLC analysis of resveratrol (100 μM) oxidation by tyrosinase. Sampling time was chosen at 0 min (A), 15 min (B), 30 min (C), 45 min (D) and 60 min (E). HPLC operating conditions were as follows; Deverosil ODS-UG-5 (Nomura Chemical, CO., LTD, Seto-Shi, Aichi, Japan). Solvent; 25% MeCN/H_2O containing 0.2% TFA, Flow rate 1.0 mL/min, detection; UV at 280 nm, 0.04 range, injected amount; 25 μL. Peak ***a*** represents resveratrol. Peaks ***b***, ***c***, and ***d*** indicate oxidation products of resveratrol.

reaction products (peaks ***b***, ***c***, ***d***) were observed; however, these oxidation product(s), unfortunately, were not stable enough to identify the structure as Espin and Wichers described in their previous investigation.[33] The effects of the oxidation of resveratrol on mushroom tyrosinase is poorly understood; hence, further investigation was conducted.

3.2. Effects of resveratrol on L-tyrosine oxidation

L-Tyrosine is an endogenous substrate of tyrosinase in nature, and the examination of the effect of resveratrol on tyrosinase-catalyzed L-tyrosine oxidation is significant in the biological point of view. Tyrosinase activities were examined with three different approaches: UV–vis spectrum at 475 nm, oxygen consumption, and HPLC. Dopachrome formation was measured with UV–vis spectrum at 475 nm, and resveratrol did not exhibit inhibitory effect on tyrosinase-catalyzed formation of dopachrome for 60 min in this assay (Fig. 3A). The formations of dopachrome (absorbance at 475 nm) were significantly increased in a concentration-dependent manner (curves 2, 3, and 4 in Fig. 3A) with the addition of resveratrol. The shape of the sample curves was significantly different from that of the control. With resveratrol, the absorbance at 475 nm was sharply increased within 20 min and then decreased, while the curve without resveratrol (DMSO control) was almost linearly increased during the entire reaction period. The extension of lag phase, which is commonly observed when compounds act as a monophenol analogue, was also observed in the case of resveratrol. Resveratrol dose-dependently extended the lag phase of oxidation of L-tyrosine. Tyrosinase activity was measured by monitoring the consumption of another substrate, oxygen. Oxygen consumption was not suppressed with the addition of resveratrol but was rather enhanced in a concentration-dependent manner (Fig. 3B). After the addition of resveratrol, the rate of oxygen consumption, interestingly, was accelerated; however, the oxygen consumption after 20 min became a stationary phase. Both UV and oxygen consumption results indicated resveratrol to be oxidized during the reaction. However, it appeared that, after 20 min, tyrosinase activities were inhibited. Thus, resveratrol requires the time (20–30 min in this condition) to inhibit tyrosinase. Subsequently, L-tyrosine consumption was monitored with HPLC analysis (Fig. 4). L-Tyrosine was time-dependently decreased without the addition of resveratrol (control) while L-tyrosine consumption was significantly suppressed with 100 μM resveratrol. Almost all L-tyrosine oxidation was diminished with the addition of resveratrol for 60 min. Furthermore, the effect of resveratrol on L-tyrosine was measured with consecutive UV–vis spectra assay (Fig. 5). The formation of dopachrome and dopaquinone was corresponding to the evolution of the peak at 475 and 350 nm, respectively, during the tyrosinase-catalyzed oxidation of L-tyrosine. The incubation of L-tyrosine and tyrosinase allowed to elevate the peak at 475 and 375 nm, and decreased the peak at 280 nm

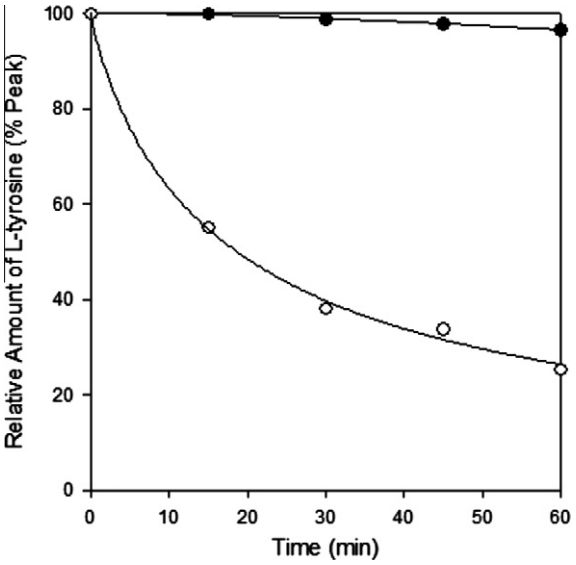


Figure 4. HPLC analysis of L-tyrosine (100 μM) oxidation by tyrosinase in absence (○) or presence (●) of 100 μM resveratrol. Sampling time was chosen at 0 min, 15 min, 30 min, 45 min, and 60 min. HPLC operating conditions were as follows; Develosil ODS-UG-5 (Nomura Chemical, CO., LTD, Seto-Shi, Aichi, Japan). Solvent; 7% MeCN/H_2O containing 0.2% TFA, flow rate 1.0 mL/min, detection; UV at 280 nm, 0.02 range, injected amount; 25 μL. SigmaPlot (Systat Software, Inc.) was used for curve fitting.

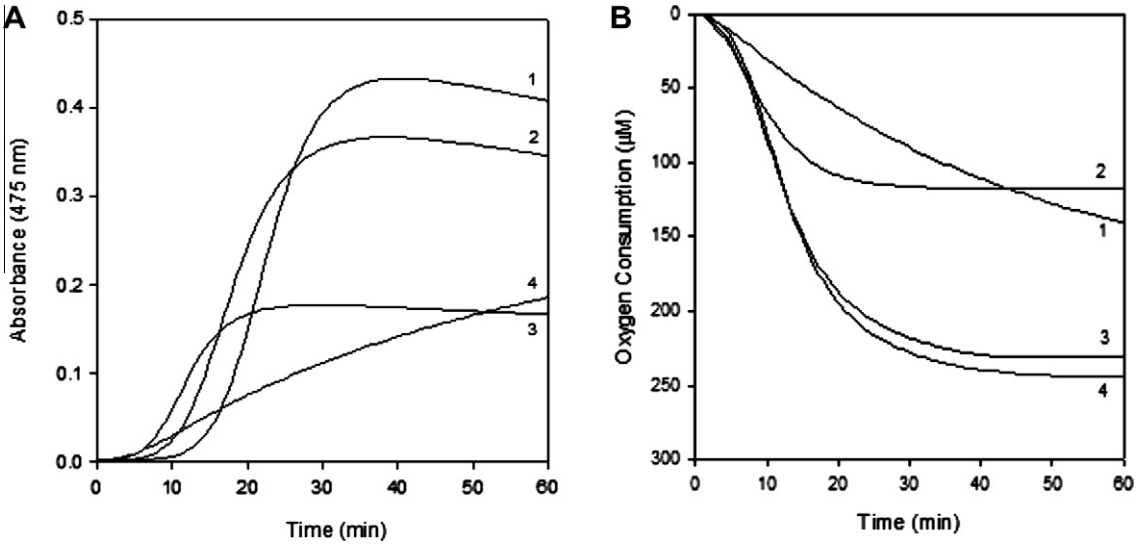


Figure 3. (A) UV–vis spectra at 475 nm obtained in oxidation of 100 μM L-tyrosine by mushroom tyrosinase in presence of resveratrol for 60 min. Concentrations of resveratrol were selected at 1000 μM (1), 500 μM (2), and 100 μM (3). Line 4 represents oxidation of L-tyrosine by mushroom tyrosinase in absence of resveratrol. (B) Oxygen consumption of oxidation of L-tyrosine (100 μM) by mushroom tyrosinase in presence of resveratrol for 60 min. The concentrations of resveratrol were 100 μM (2), 500 μM (3), and 1000 μM (4). Line 1 represents the oxygen consumption of oxidation of 100 μM L-tyrosine by mushroom tyrosinase in absence of resveratrol.

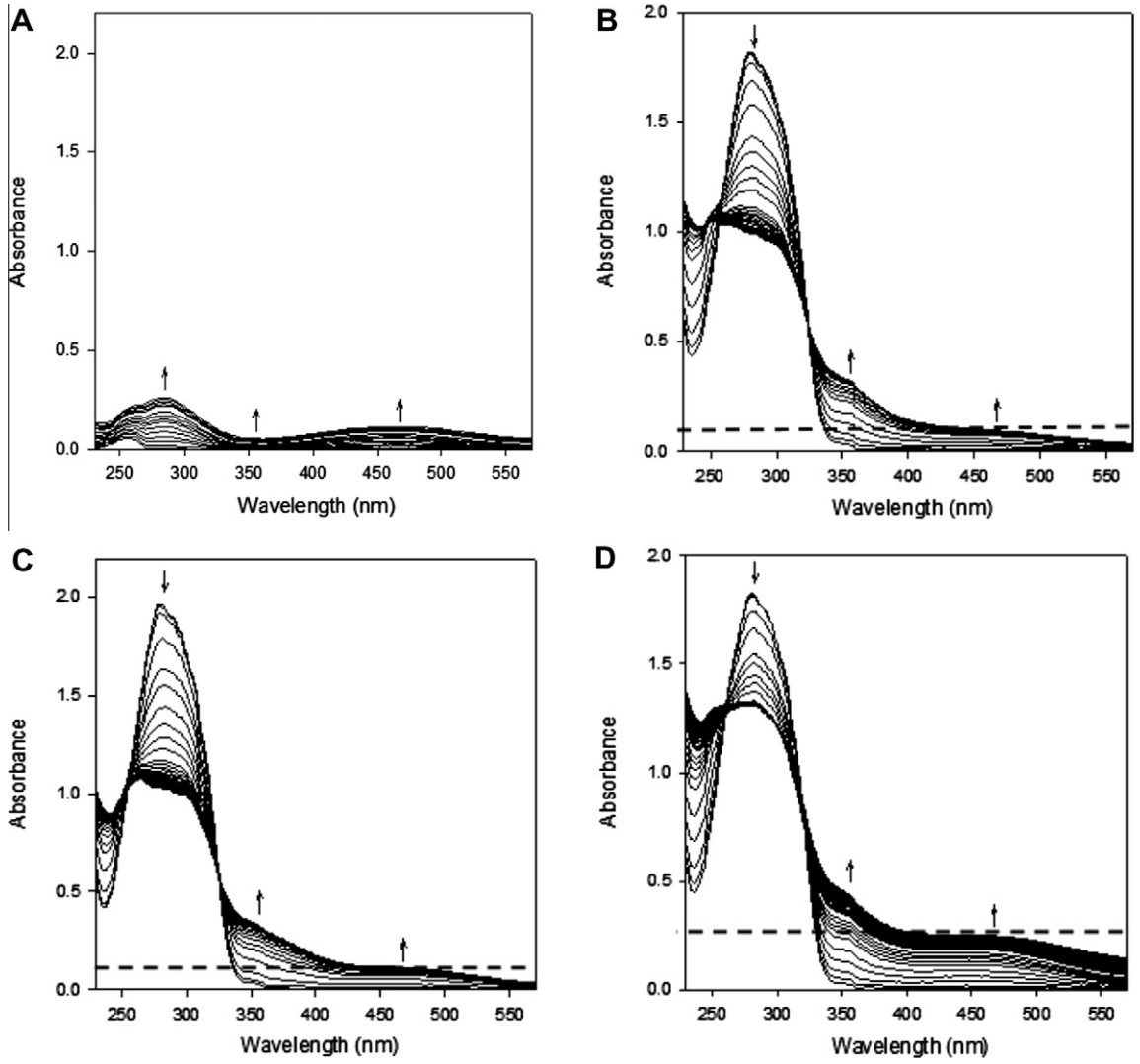


Figure 5. Consecutive spectra obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in absence (A) or presence (C) of 100 μM resveratrol for 60 min. B represents the oxidation of 100 μM resveratrol by mushroom tyrosinase. D indicates that simulated spectrum that is obtained from the sum of spectrum of L-tyrosine oxidation and of resveratrol oxidation (i.e., A + B). Scan speed was at 2 min intervals for 30 s. the arrows (↑) designate the evolution of the peak.

(Fig. 5A). Interestingly, tyrosinase-assayed resveratrol showed similar spectra to the case of L-tyrosine and resveratrol (Fig. 5B and C). In Fig. 5B, 100 μM resveratrol (280 nm) was quickly oxidized and corresponding quinone compounds (320 nm) were formed in first 10 min; however, the spectra also showed further oxidation of oxidized products of resveratrol (460 nm region). Figure 5C indicated that even though L-tyrosine was contained in the mixture, it seemed that dopachrome corresponding to L-tyrosine oxidation was not formed. This phenomenon was further supported by creating Figure 5D, which represented that simulated spectra which created from the addition of spectra A and B. In simulated graph, the dopachrome formation was about 0.25 in absorbance unit; however, the actual observed absorbance in Figure 5C was about 0.125. This suggested that most of dopachrome formation was suppressed by the addition of resveratrol as well as the result of HPLC analysis.

3.3. Effects of resveratrol on L-DOPA oxidation

To find the inhibitory mechanism of resveratrol on tyrosinase activity, the effect of resveratrol on the tyrosinase-catalyzed L-DOPA oxidation was examined. L-DOPA was used as a substrate in order to determine the effect of resveratrol on diphenolase activities of tyrosinase. Tyrosinase activity against L-DOPA was also measured with three different methods described above. With UV–vis spectra at 475 nm, resveratrol did not show significant inhibition on L-DOPA oxidation but rather enhanced as well as in the result observed with L-tyrosine with 30 min of reaction period (Fig. 6A). In Figure 6A, resveratrol was oxidized and newly oxidized compound(s) was observed at 475 nm (curve 3), and the mixture of L-DOPA and resveratrol significantly enhanced the absorbance at 475 nm (curves 1 and 2). The similar results were obtained with the oxygen consumption assay (Fig. 6B). In the case of tyrosinase-catalyzed 'resveratrol' oxidation, the induction phase was observed in both UV–vis spectra and oxygen consumption assays; however, this lag phase was eliminated with the addition of L-DOPA due to the effect of a cofactor. Subsequently, the L-DOPA consumption was measured with HPLC assay. The rate of oxidation of L-DOPA became slower when resveratrol was added, but at the end of the reaction, most of the L-DOPA was consumed in both cases (data not shown). It should be pointed out that resveratrol was not oxidized before L-DOPA oxidation while resveratrol was oxidized before the oxidation of L-tyrosine. Thus, resveratrol acted as a monophenol substrate and resveratrol itself did not inhibit tyrosinase, but the oxidized product(s) potentially inhibited tyrosinase activities.

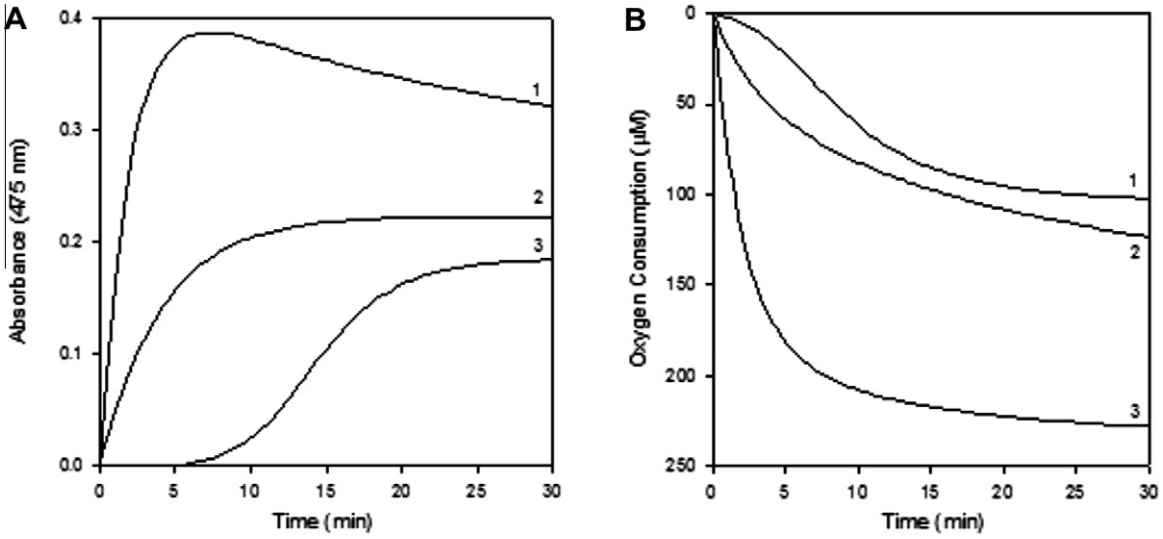


Figure 6. (A) UV–vis spectra at 475 nm obtained in oxidation of 100 μM L-DOPA by mushroom tyrosinase in presence (1) or absence (2) of resveratrol for 30 min. Concentrations of resveratrol were selected at 100 μM. Line 3 represents oxidation of resveratrol by mushroom tyrosinase without L-DOPA. (B) Oxygen consumption of oxidation of L-DOPA (100 μM) by mushroom tyrosinase in absence (2) or presence (3) of resveratrol for 30 min. The concentrations of resveratrol were 100 μM. Line 1 represents the oxygen consumption of oxidation of 100 μM resveratrol by mushroom tyrosinase without L-DOPA.

3.4. Effect of preincubation on inhibitory activity of resveratrol

Based on the results of the effect of resveratrol on L-tyrosine or L-DOPA oxidation, oxidized product(s) of resveratrol may be the key of inhibitory activity against tyrosinase. Hence, the effect of resveratrol after its oxidation was examined; preincubational experiments were performed for this purpose. First, 100 μM resveratrol was oxidized with tyrosinase for 30 min (preincubational process), and then either 100 μM L-tyrosine or L-DOPA was added to the mixture to measure tyrosinase activity. Tyrosinase activity on L-tyrosinase oxidation was examined first. As expected, L-tyrosine oxidation was not observed in both UV–vis and oxygen consumption assays (Fig. 7). In Figure 7A, dopachrome was formed with DMSO while it was not with resveratrol, and the activity of tyrosinase on L-tyrosinase oxidation was nearly lethal. A similar result was obtained from the oxygen consumption assay (Fig. 7B). Secondly, the preincubational effect was tested on tyrosinase-catalyzed L-DOPA oxidation. While without preincubation, resveratrol did not inhibit L-DOPA oxidation, with preincubation, resveratrol strongly suppressed L-DOPA oxidation (Fig. 8). Dopachrome formation in 30 min was strongly suppressed (Fig. 8A) as well as the oxygen consumption (Fig. 8B). Almost 90–100% of L-DOPA oxidation was inhibited with this preincubational experiment. These experiment results were further supported with HPLC analysis (Fig. 9). In Figure 9-1, L-DOPA (peak ***a***; t_R = 6.2 min) was quickly oxidized by tyrosinase (up to 95% of L-DOPA) and dopachrome (peak ***b***; t_R = 4.8 min) was newly formed. However, after the 30 min of preincubation, only 10% of L-DOPA was oxidized for 30 min, and a small peak of dopachrome was observed (Fig. 9-2). Thus, it is logical to conclude that resveratrol undergoes enzymatic activation prior to the inhibition of tyrosinase (k_{cat} type inhibition).

3.5. Recovery from the preincubational effects

Further detail of the mechanism of inhibition of resveratrol was examined by using L-cysteine. The inhibitory effect on

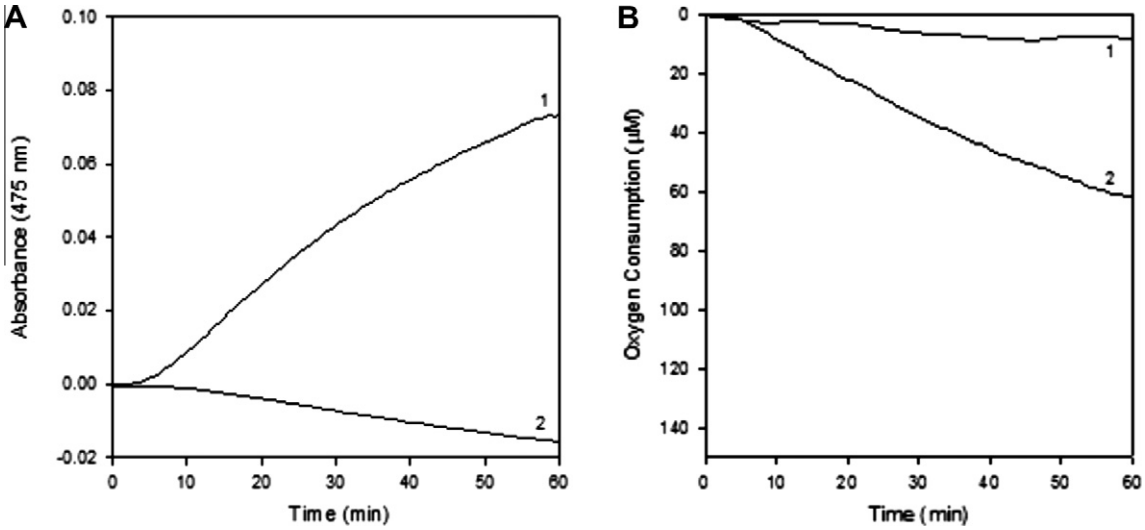


Figure 7. (A) UV–vis spectra at 475 nm obtained in oxidation of 100 μM L-tyrosine by mushroom tyrosinase in absence (1) or presence (2) of resveratrol for 60 min after 30 min of preincubation. Concentrations of resveratrol were selected at 100 μM. (B) Oxygen consumption of 100 μM L-tyrosine oxidation by mushroom tyrosinase in presence (1) or absence (2) of resveratrol for 60 min after 30 min of preincubation. Concentrations of resveratrol were selected at 100 μM.

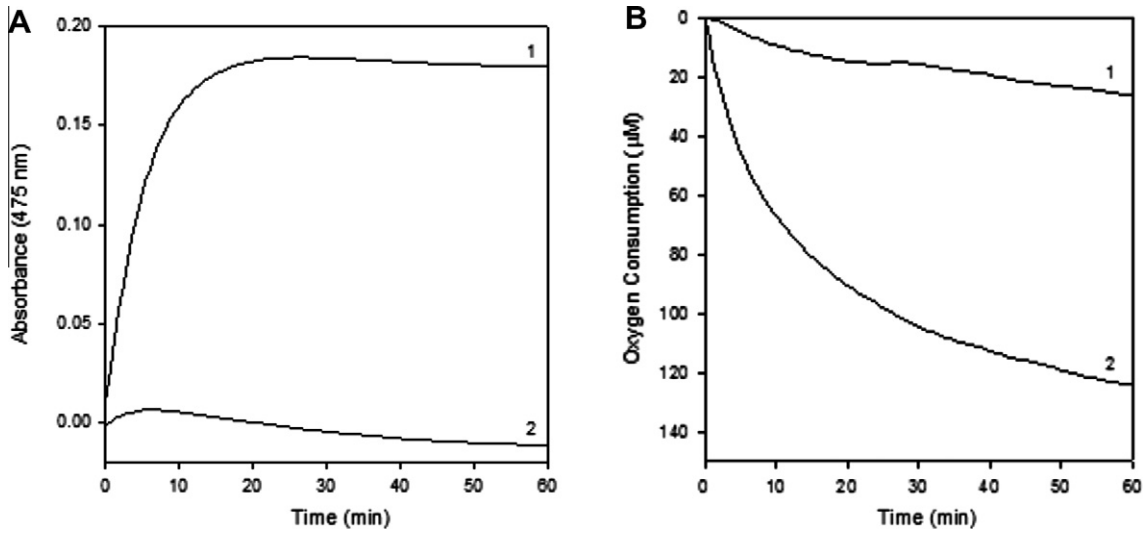


Figure 8. (A) UV–vis spectra at 475 nm obtained in oxidation of 100 μM L-DOPA by mushroom tyrosinase in absence (1) or presence (2) of resveratrol for 60 min after 30 min of preincubation. Concentrations of resveratrol were selected at 100 μM. (B) Oxygen consumption of 100 μM L-DOPA oxidation by mushroom tyrosinase in presence (1) or absence (2) of resveratrol for 60 min after 30 min of preincubation. Concentrations of resveratrol were selected at 100 μM.

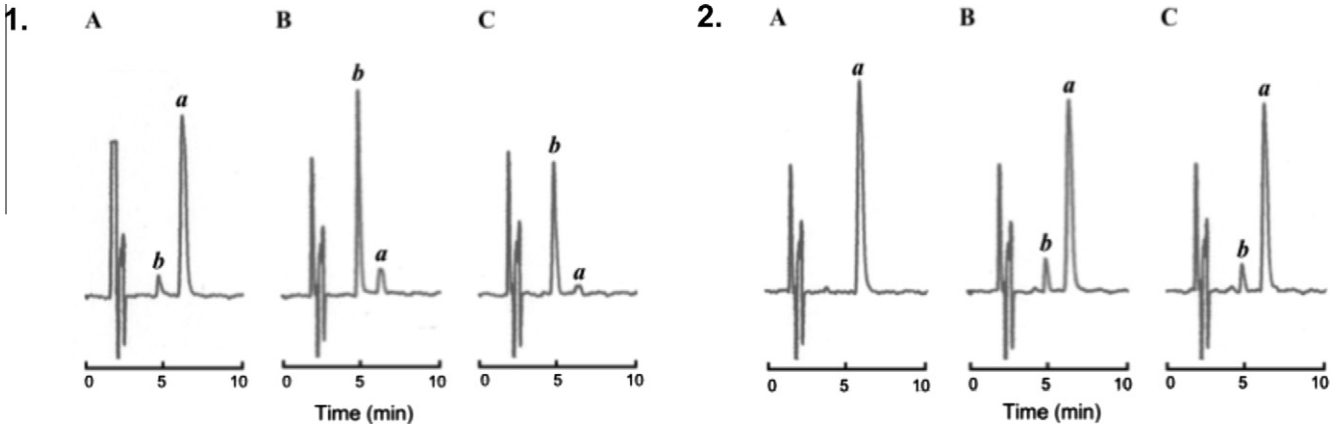


Figure 9. HPLC analysis of L-DOPA (100 μM) oxidation by mushroom tyrosinase in absence (1) or presence (2) of 100 μM resveratrol after 30 min of preincubation. Sampling time was chosen at 0 min (A), 15 min (B), 30 min (C). HPLC operating conditions were as follows; Develosil ODS-UG-5 (Nomura Chemical, CO., LTD, Seto-Shi, Aichi, Japan). Solvent; 7% MeCN/H_2O containing 0.2% TFA, flow rate 1.0 mL/min, detection; UV at 280 nm, 0.02 range, injected amount; 25 mL. Peaks ***a*** and ***b*** represent L-DOPA and dopachrome, respectively.

tyrosinase-catalyzed L-DOPA oxidation was enhanced with 30 min of preincubation; however, the effect was reduced with the addition of 200 μM L-cysteine (Fig. 10). About 20% of inhibition was reduced by introducing 200 μM L-cysteine (curves 1 and 2 in Fig. 10). In the presence of resveratrol, L-DOPA oxidation was not observed without L-cysteine (curve 1 in Fig. 10). This suggested that L-cysteine disrupted the inhibitory effect of resveratrol on tyrosinase activity. During the oxidation of resveratrol, resveratrol is converted to *o*-quinone. L-Cysteine undergoes nucleophilic addition to react with the quinone, which leads to the removal of the metabolite(s) of resveratrol. Thus, this reduction of inhibitory effect with the addition of L-cysteine explains that inhibitory action of resveratrol is due to the formation of reactive electrophile(s) such as *o*-quinone.

3.6. Resveratrol as a melanogenesis Inhibitor on B16-F10 melanoma cells

From the results of cell-free investigations, resveratrol showed the potent inhibitory effect on tyrosinase activity. Thus, the investigations were extended to cellular experiments. The initial goal was to test whether resveratrol inhibits melanogenesis in cultured melanocytes without affecting cell growth. Hence, their cell viability was examined first. In this regard, cell viability was determined on the third day for melanocytes using both trypan blue dye exclusion and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays. The same result was usually observed by both assays, but the concentration leading to 50% viable cells lost (IC_{50}) was established by trypan blue assay for steady comparison purpose. The specificity of melanogenesis inhibition was assessed by dividing the melanin content by the number of cells determined by trypan blue exclusion. The appropriated concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay.

Cytotoxic and antimelanogenic effects of resveratrol were identified. The highest examined concentration of resveratrol was 200 μM. Resveratrol slightly suppressed cell viability in a dose-dependent manner (Fig. 11A), and IC_{50} was not observed up to 200 μM. About only 10% of cell viability was suppressed with 200 μM resveratrol. The cell viability above 200 μM was significantly different (P <0.01) from the control. Total melanin production above 25 μM was significantly suppressed (P <0.01) in a

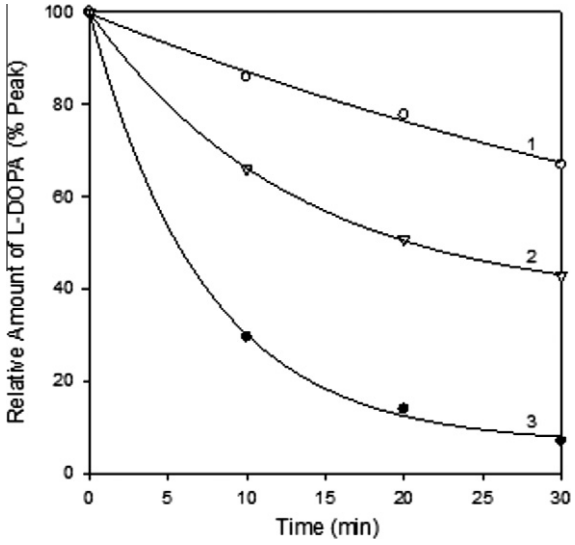


Figure 10. HPLC analysis of L-DOPA (100 μM) oxidation by mushroom tyrosinase in presence of 100 μM resveratrol with or without L-cysteine after 30 min of preincubation. Line 1 and 2 represent that the tyrosinase-catalyzed oxidation of L-DOPA in presence of 100 μM resveratrol and 0 μM (1) or 200 μM (2) L-cysteine. Line 3 indicates L-DOPA oxidation by tyrosinase in absence of both resveratrol and L-cysteine. Sampling time was chosen at 0 min, 10 min, 20 min, and 30 min. HPLC operating conditions were as follows; Develosil ODS-UG-5 (Nomura Chemical, CO., LTD, Seto-Shi, Aichi, Japan). Solvent; 10% MeCN/H_2O containing 0.2% TFA, flow rate 1.0 mL/min, detection; UV at 280 nm, 0.02 range, injected amount; 25 mL. SigmaPlot (Systat Software, Inc.) was used for curve fitting.

concentration-dependent manner (Fig. 11B). It came out that the total melanin production was reduced without affecting the cell growth; hence, cellular melanin production is dose-dependently suppressed. The significant difference from the control (P <0.01) was observed when 200 μM resveratrol was applied (Fig. 11C). Furthermore, other stilbenoids, namely, *trans*-pinosylvin (**2**), *cis*-pinosylvin (**3**), dihydropinosylvin (**4**) were tested for the comparisons (data not shown). None of these compounds suppressed cellular viability in a concentration-dependent manner. Only *trans*-pinosylvin showed the significant difference is observed at 400 μM. Cellular melanin content was not suppressed but rather increased with the addition of *trans*-, *cis*- or dihydropinosylvin. Cellular morphological changes of resveratrol-treated melanoma cells were also microscopically observed (data not shown). The cellular morphology of control (DMSO treated) cells was almost exactly the same as that of resveratrol-treated cells. Thus, resveratrol is a unique potentiated melanogenesis inhibitor with less/no cytotoxic effects.

4. Discussion

Tyrosinase inhibitor can be used in variety of ways including cosmetic products and food antibrowning reagents. Because of this purpose, several antimelanogenic reagents have been developed and discovered nowadays. However, only a few of the inhibitors have been able to introduce and use because of the problems in cytotoxicity (affecting the cell growth), selectivity, solubility, and stability. Resveratrol is a naturally occurring polyphenol, and is commonly taken in foods or beverages. Hence, it is no doubt that resveratrol is more applicable as a melanogenesis drug than any other synthetic chemicals (if the biological effects of resveratrol are inappropriate). Based on the data obtained, it appeared that resveratrol inhibits tyrosinase activities through the mechanism of k_{cat} type inhibition. According to Robert Rando, k_{cat} inhibitors can be constructed to possess latent reactive grouping that is selectively activated by the target enzyme at its active site.[23] Usually,

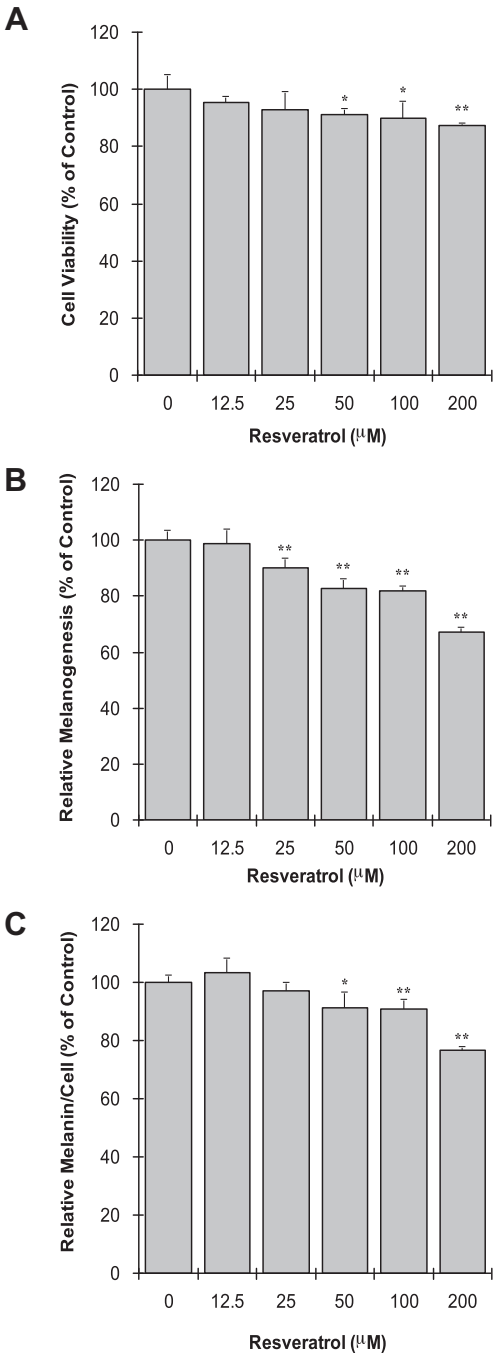
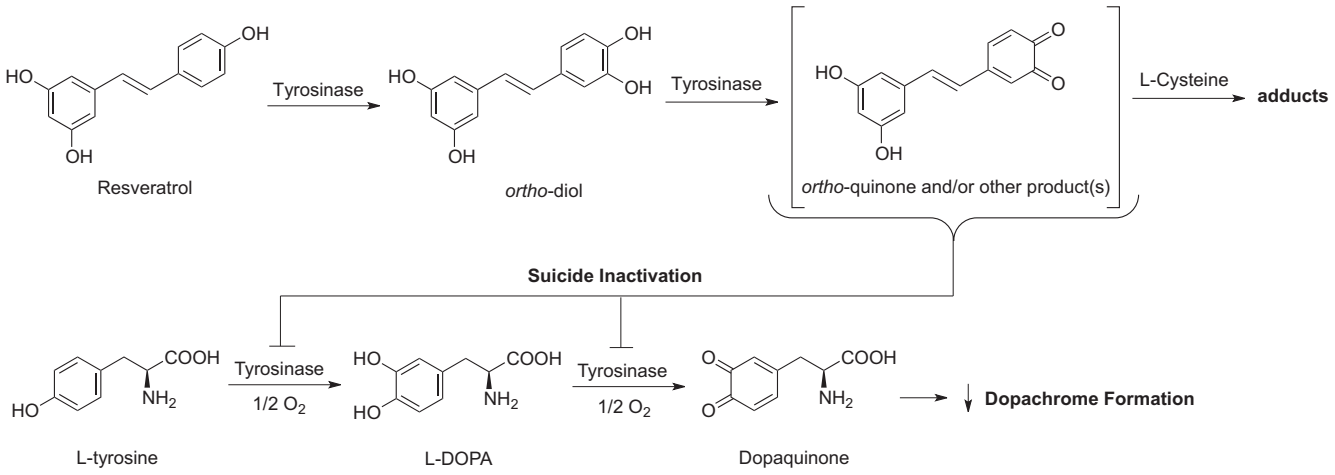


Figure 11. (A) Viabilities of B16-F10 melanoma cells following treatment with resveratrol for 72 h; data are expressed as percentage of the number of viable cells observed with the control, and each column represents the mean ± SD of at least 4 determinations. (B) Total melanin content in B16 melanoma cells following treatment with resveratrol for 72 h; data are expressed as percentage of melanin content per well observed with the control, and each column represents the mean ± SD of 4 determinations. (C) Cellular melanin content in B16 melanoma cells following treatment with resveratrol for 72 h measured as percentage of melanin content per cell observed with the control, and each column represents the mean ± SD of 4 determinations. The statistical significance of differences was evaluated using Student's or Welch's t-test. Significantly different from the control value: $^{*}p$ <0.05, $^{**}p$ <0.01.

highly reactive product(s) are synthesized, and once generated, they react with the enzyme, which leads to its irreversible inhibition. Thus, activation of resveratrol thorough the oxidation by tyrosinase is the key process of the mode of action. Interestingly, resveratrol-4-*O*-methyl ether showed less inhibitory activity



Scheme 1. Potentiated mechanism of k_{cat}-type inhibition by resveratrol on tyrosinase activity.

against mushroom tyrosinase and B16 melanoma cells,[21] which is confirmed by our conclusions. This helps understanding that 4′-hydroxyl group is essential for the inhibition. Furthermore, based on the previous studies, resorcinolic moiety has high inhibitory potency to tyrosinase while 4-hydroxyl group is the subject of the oxidation, for instance, arbutin (**5**) or methyl *p*-coumarate (**6**).[34–38] As Espin studied using polyphenol oxidase (PPO),[33] resveratrol is converted to *ortho*-diol and further oxidized to quinone-derivatives (Scheme 1). As they commented in their previous study, oxidation product(s) of resveratrol was unstable, and it could not reach to the steady state. Their result was confirmed by our HPLC, UV–vis and oxygen consumption assays (Figs. 2, 5B, and 6B line 3). The instability of oxidation product(s) also suggested the mechanism of suicide inhibition by resveratrol. Comparing the data from L-tyrosine and L-DOPA, resveratrol is relatively easy to be oxidized; the order of the rate of oxidation is the following: L-DOPA > resveratrol > L-tyrosine. Hence, L-DOPA oxidation was not inhibited by resveratrol without preincubation. It is not clear if the suicide inactivation of tyrosinase is due to one of either (1) the formation of reactive oxygen species that attack on the active site of enzyme,[39] (2) 'cresolase-type mechanism of tyrosinase' to inactivate active copper of tyrosinase to be a copper(0),[40] or (3) formation of oxidized product(s) of resveratrol such as quinone.[41] However, in the case of resveratrol, it seems the last case (case 3) is true. Figure 10 represented that the addition of L-cysteine prevents the tyrosinase from its inactivation. L-Cysteine reacts with quinone (or maybe other metabolites) of resveratrol and diminished the inactivation. Furthermore, Kubo et al. have suggested the mechanism of melanogenesis inhibition on B16 mouse melanoma cells due to methyl *p*-coumarate,[35] a potent melanin formation inhibitor, is caused by the metabolite(s) of the compound but not by the compound itself. Based on our results and the structural similarities, it is understandable that resveratrol acts as a suicide inhibitor.

Antioxidants have variety of activities, including direct quenching of reactive oxygen species (ROS), inhibition of enzymes involved in the production of ROS, chelation of low-valent metal ions such as Fe^{2+} or Cu^{2+}, and regeneration of membrane-bound antioxidants such as α-tocopherol.[42] Resveratrol is a known antioxidant and extensive investigations have been done. Including radical scavenging activities,[43] in several reports, resveratrol induces cardioprotective effect, anti-aging, apoptotic effect and anti-carcinogenesis.[11,14,19,44] Antimelanogenic effect could be added to one of the biological function of resveratrol and this natural polyphenol could be developed as a multifunctional drug.

Safety is the primary concern for any purposes including cosmetic products or chemotherapeutic reagents. Resveratrol is oxidized by tyrosinase and the metabolite(s) would inhibit tyrosinase activity and reduce the cellular melanin content; however, the effects/functions of these metabolites to other biological systems are still unclear. Resveratrol is found in many natural sources, and hence, it may be the alternate choice to non-natural chemical reagents. Despite clarification of the mechanism of anti-tyrosinase effect, the detail of inhibitory mechanism of cellular melanogenesis by resveratrol is still under investigations.

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