

Effects of Salicylic Acid on Mushroom Tyrosinase and B16 Melanoma Cells

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Salicylic acid slightly inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase noncompetitively without being oxidized. In contrast, 4-hydroxybenzoic acid did not inhibit this enzymatic oxidation if a longer reaction time was observed, although it suppressed the initial rate of the oxidation to a certain extent. Neither acid showed noticeable effects on cultured murine B16-F10 melanoma cells except weak cytotoxicity.

Key words: Salicylic Acid, Tyrosinase, B16-F10 Melanoma Cells

Introduction

In our continuing search for tyrosinase inhibitors from plants, anacardic acids, 6-pentadecenylsalicylic acids, were characterized as active principles (Kubo *et al.*, 1994). During the study of their inhibition kinetics, we became aware that the pentadecenyl group plays an important role. The enzyme first quickly and reversibly binds anacardic acids and then undergoes a slow irreversible course to inactivation. The pentadecenyl side chain in anacardic acids seems to be related to the inhibitory activity, presumably by interacting with the hydrophobic domain close to the binuclear copper active site in the enzyme. Since anacardic acids are salicylic acid (**1**) derivatives with a pentadecenyl side chain, their inhibitory activity needs to be compared with that of salicylic acid. Salicylic acid was previously described to be a slow and reversible agent with residual enzyme activity. The inhibition kinetics of salicylic acid analyzed by Lineweaver-Burk plots showed that **1** is a noncompetitive inhibitor (Kubo *et al.*, 1994). In the previous experiments, the enzyme activity was monitored by measuring the dopachrome formation at 475 nm using the commercial tyrosinase. Dopachrome is relatively stable but is gradually oxidized further (Mayer *et al.*, 1966), and hence, the spectrophotometric method measures only the very initial rate of dopachrome formation. Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen. The previous assay was carried out in air-saturated aqueous solutions, and hence,

oxygen was not treated as a substrate quantitatively. This prompted us to reinvestigate the previous tyrosinase inhibition kinetics from a different point of view using purified tyrosinase. The aim of this paper is to report the tyrosinase inhibitory action of salicylic acid studied mainly by the polarographic method linked to consecutive spectral and HPLC analyses (Ha *et al.*, 2005). In addition, since tyrosinase is a key enzyme in the melanin synthetic pathway, the experiment was extended to examine the effects of salicylic acid and its related compounds on murine B16-F10 melanoma cells.

Material and Methods

General procedures

General procedures were the same as in previous works (Kubo *et al.*, 1994; Kubo and Kinst-Hori, 1998; Nihei and Kubo, 2003). HPLC analysis was performed on an Eyela LPG-1000 instrument with an Eyela UV-7000 detector (Tokyo Rikakikai, Tokyo, Japan) and a Shiseido Capcell Pak C-18 column (5 μ m, 4.4 mm \times 250 mm; Shiseido, Tokyo, Japan).

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and MTT cell proliferation assay kit were purchased from ATCC (Manassas, VA, USA). Anacardic acid (C_{15:1}) was available from our previous work (Kubo *et al.*, 1986). Salicylic acid, salicylaldehyde, salicyl alcohol, 4-hy-

droxybenzoic acid, and kojic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). L-3,4-Dihydroxyphenylalanine (L-DOPA) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Enzyme assay

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 (Espín and Wichers, 1999). The current experiment was subjected to use the purified tyrosinase, but basically the same result was also observed using the commercial tyrosinase. Although mushroom tyrosinase differs somewhat from other sources, this fungal source 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 and SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA) at 30 °C. All samples were first dissolved in DMSO at 150 mM and used for the experiment with dilution. The final content of DMSO in the test solution is always 3.3%. The assay was performed as previously reported (Kubo and Kinst-Hori, 1998, 1999) with slight modifications. First, 0.06 ml of a 5.0 mM L-DOPA or L-tyrosine aqueous solution was mixed with 0.6 ml of 0.25 M phosphate buffer (pH 6.8) and 2.14 ml water and incubated at 30 °C for 10 min. Then, 0.1 ml of the sample solution and 0.1 ml of the same phosphate buffer solution of the mushroom tyrosinase (1 µg/ml) were added in this order to the mixture. Consecutive spectra from 220 to 600 nm were immediately recorded at 1 min intervals for 30 min in L-DOPA and at 5 min intervals for 60 min in L-tyrosine, respectively. To obtain IC₅₀ values, the final assay concentration of the substrate (L-DOPA) was adjusted to 0.5 mM.

Oxygen consumption assay

The reaction mixture consisting of 0.6 ml of 0.25 M phosphate buffer (pH 6.8), 1.9 ml of water and 0.1 ml of the test sample DMSO solution was first incubated at 30 °C for 5 min. Then, 0.1 ml of 0.05 M phosphate buffer solution of mushroom ty-

rosinase (0.2 mg/ml) was added and oxygen consumption was measured with an OBH 100 oxygen electrode and an oxygraph equipped with a YSI 5300 water-jacket chamber (all from Yellow Springs Instruments Co., Yellow Springs, OH, USA) maintained at 30 °C for 20 min. Catalytic amount (0.01 mM) of L-DOPA was added after 5 min. The oximeter was calibrated with air (100%) and sodium dithionite (0%).

Cell culture

B16-F10 mouse melanoma cells (CRL-6475) were obtained from ATCC 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% CO₂ for about 24 h before the chemical treatment. Each chemical combination was applied in duplicate with a final content of 0.1% DMSO, and treated cells were cultured for 72 h before assays.

Cell viability assays

Cell viability was determined by trypan blue exclusion using a hemocytometer. After overnight incubation of cells, viability was also determined by the MTT cell proliferation assay. Both bioassays basically provided the same results but the concentration leading to 50% viable cells lost (IC₅₀) was established by the 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). It should be noted that cell viability of salicylic acid and 4-hydroxybenzoic acid was estimated by microscopic observation.

Trypan blue method

Cells were washed with PBS and dispersed with trypsinization, and 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.

MTT method

Cell viability was examined also by the MTT cell proliferation kit following the instructions of the

manufacturer. MTT is reduced by mitochondrial dehydrogenases in living cells to a blue-magenta colored formazan precipitate. Briefly, 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% CO₂ for 16 to 24 h. At the end of the period, 10 μ l of MTT reagent were added to each well, which was then incubated at 37 °C in 5% CO₂ for 4 h. Then, 100 μ l of detergent reagent were added to each well. The plate was kept at room temperature in the dark for 2 h, and relative amount of MTT reduction was determined based on the absorbance at 570 nm using a SpectraMax Plus Microplate spectrophotometer and SoftMax Pro software (Molecular Devices).

Melanin assay

The melanin content was determined as previously described (Kageyama *et al.*, 2004; Venkatasamy *et al.*, 2004) with minor modification. Cells were washed with PBS, harvested by trypsinization, and centrifuged for 10 min at 1500 \times 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).

Statistical analysis

The data were evaluated by either Student's or Welch's *t*-test after examining the variances using F test. *p* < 0.01 was considered to be statistically significant.

Results

Salicylic acid (2-hydroxybenzoic acid) (**1**) (see Fig. 1 for structures) exhibited a concentration-dependent inhibitory effect on the tyrosinase-catalyzed oxidation of L-DOPA. As the concentration of salicylic acid increased, the enzyme activity rapidly decreased. The remaining enzyme activity was about 30% when the concentration of salicylic acid reached 4 mM (Fig. 2). The inhibitory concentration leading to 50% activity lost (IC₅₀) was estimated as 1.0 mM, *i.e.* **1** is about 4-fold more potent compared to 3.9 mM obtained with the commercial tyrosinase. In contrast to anacardic acids, the inhibitory activity did not increase when the enzyme was preincubated for 10 min with salicylic acid. The inhibition kinetics of salicylic acid analyzed by

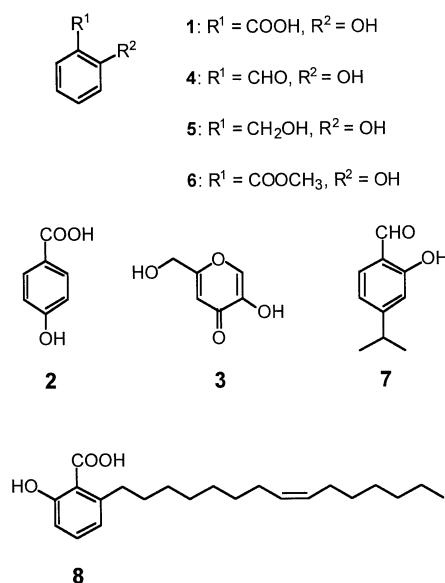


Fig. 1. Chemical structures of salicylic acid (**1**) and its related compounds (**2–8**).

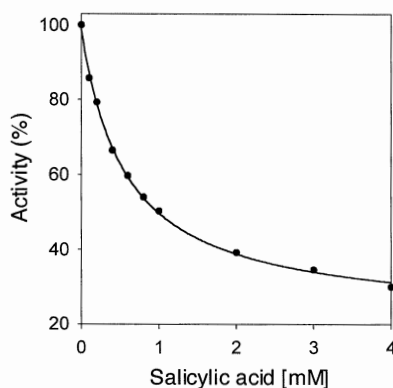


Fig. 2. Inhibitory effect of salicylic acid on the oxidation of L-DOPA catalyzed by tyrosinase.

Lineweaver-Burk plots indicated that salicylic acid is a noncompetitive inhibitor as previously reported (Kubo *et al.*, 1994). The equilibrium constant for inhibitor binding, *K*_{IS}, was obtained as 0.9 mM from Dixon plots. 4-Hydroxybenzoic acid (**2**) was also examined for comparison. Similar results were obtained with **2**. In brief, the IC₅₀ value was established as 2.0 mM. The inhibition kinetics of 4-hydroxybenzoic acid analyzed by Lineweaver-Burk plots indicated 4-hydroxybenzoic acid to be a competitive inhibitor (Kubo *et al.*, 2003). The inhibition by 4-hydroxybenzoic acid is a reversible

reaction. The inhibitory activity did not increase when the enzyme was preincubated with 4-hydroxybenzoic acid but without L-DOPA for 10 min. The current experiments were done using the purified tyrosinase, but basically the same results were observed with commercial tyrosinase.

The effects of salicylic acid on the tyrosinase-catalyzed oxidation of L-DOPA were examined.

Salicylic acid slightly suppressed the oxygen consumption when the reaction mixture consisting of the enzyme (1 $\mu\text{g}/\text{ml}$), 1.0 mM of salicylic acid and 0.1 mM of L-DOPA was incubated for 60 min. The oxygen consumption increased with increasing time. Salicylic acid suppressed more the oxygen consumption after 60 min, and the amount of oxygen consumed after 60 min was approximately

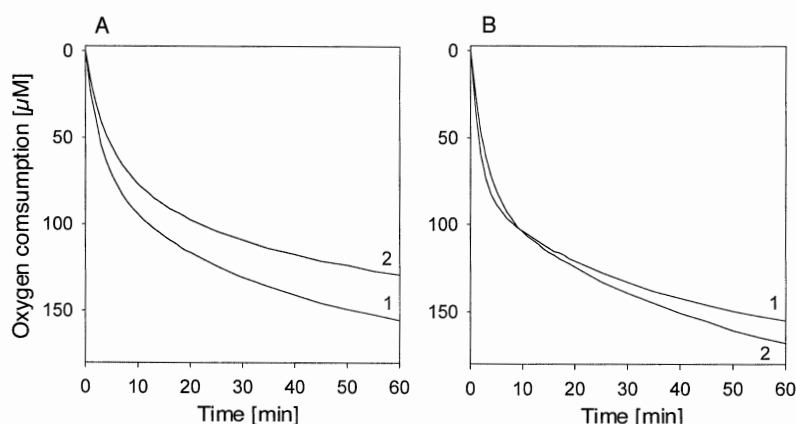


Fig. 3. (A) Oxygen consumption during the oxidation of L-DOPA alone (0.1 mM, line 1) and in the presence of salicylic acid (1.0 mM, line 2) by tyrosinase for 60 min. (B) Oxygen consumption during the oxidation of L-DOPA alone (0.1 mM, line 1) and in the presence of 4-hydroxybenzoic acid (1.0 mM, line 2) by tyrosinase for 60 min.

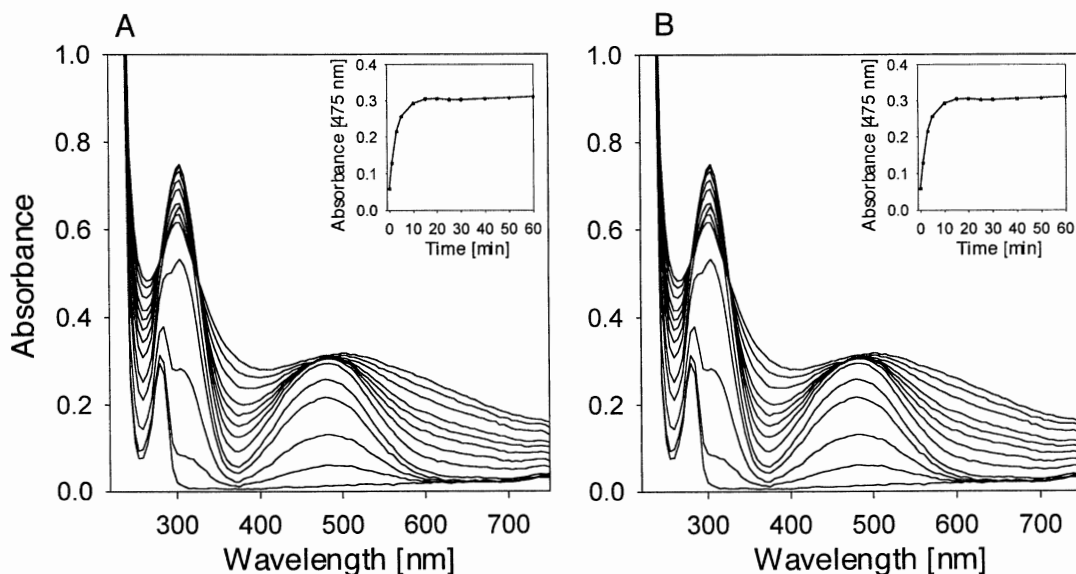


Fig. 4. (A) Consecutive spectra obtained during the oxidation of L-DOPA (0.1 mM) in the presence of salicylic acid (1.0 mM) catalyzed by tyrosinase. Scans were recorded at 0, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min. The inset shows the change in absorbance at 475 nm. (B) Consecutive spectra obtained in the oxidation of L-DOPA (0.1 mM) catalyzed by tyrosinase. Scans were recorded at 0, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min. The inset shows the change in absorbance at 475 nm.

20% less compared with the control (Fig. 3A). In the case of 4-hydroxybenzoic acid, the amount of oxygen consumed after 60 min was not significantly different from the control (Fig. 3B).

Subsequently, the consecutive spectrum was measured using 5 min intervals for 60 min under the same conditions of the oxygen uptake experiment. As shown in Fig. 4A, the changes in the spectrum started in increasing a broad absorbance with the maximum at around 475 nm, corresponding to dopachrome. The change observed (inset of Fig. 4A) is similar to the control, indicating that salicylic acid did not inhibit the tyrosinase-catalyzed oxidation of L-DOPA. In other words, L-DOPA was oxidized to dopachrome even in the presence of 1 mM of salicylic acid. It should be noted, however, that the amount of enzymatically generated dopachrome was about 20% less compared to the control (Fig. 4B). In the case of 4-hydroxybenzoic acid, the change observed was similar to the control even in the presence of 2 mM of 4-hydroxybenzoic acid, indicating that L-DOPA was quickly oxidized. Thus, 4-hydroxybenzoic acid did not inhibit the tyrosinase-catalyzed oxidation of L-DOPA.

The conclusion drawn is consistent with subsequent HPLC analyses performed for the same reaction mixture in 20 min intervals. The results are illustrated in Fig. 5. The peak identified as 4-hydroxybenzoic acid remained without any change up to 60 min. On the other hand, the peak corresponding to L-DOPA disappeared within 20 min and the peak identified as dopachrome diminished gradually, similar to the control. In brief, 4-hydroxybenzoic acid did not inhibit the enzyme activity and remained in the reaction mixture without any change.

Tyrosinase is a key enzyme in the melanin synthetic pathway and therefore tyrosinase inhibitors are expected to inhibit melanin production. However, the inhibition of mushroom tyrosinase activity was reported not to correlate with that of cellular tyrosinase or melanin production in cultured melanocytes (Maeda and Fukuda, 1996). Moreover, kojic acid (**3**), a depigmenting agent known as a copper chelator, inhibits melanin formation. There seems to be a gap between the simplified cell-free experiments and cellular application. Hence, the effects of salicylic acid on melanin synthesis in cultured murine B16-F10 melanoma cells were examined.

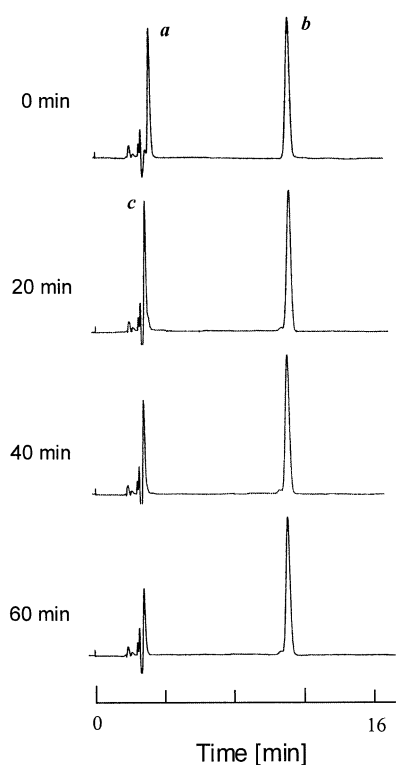


Fig. 5. HPLC analysis of the reaction medium with 1.0 mM of salicylic acid (b) and tyrosinase in the presence of 0.1 mM L-DOPA (a). Sampling time was chosen at 0, 20, 40 and 60 min, respectively. Compound c is dopachrome.

Salicylic acid did not affect cell viability when the cells were cultured with salicylic acid up to 2 mM as judged by microscopic observation. This can be explained by the knowledge that deprotonated acid is not able to enter the cells through the membrane. Thus, the highly hydrated anionic carboxy group is therefore strongly associated with the water/lipid interface of the membrane so that its passage through the lipid core is impeded by a high energy barrier. Salicylic acid did not show any cytotoxicity up to 2 mM in the same cultured melanoma cells, but weak cytotoxicity was observed at 4 mM as judged by microscopic observation. Similarly, 4-hydroxybenzoic acid did not penetrate into cells but showed inferior cytotoxicity as judged by microscopic observation. In the case of kojic acid, the melanin production was suppressed without affecting cell growth when the cells were cultured with kojic acid up to 1 mM. On the other hand, salicylaldehyde (**4**) exhibited cyto-

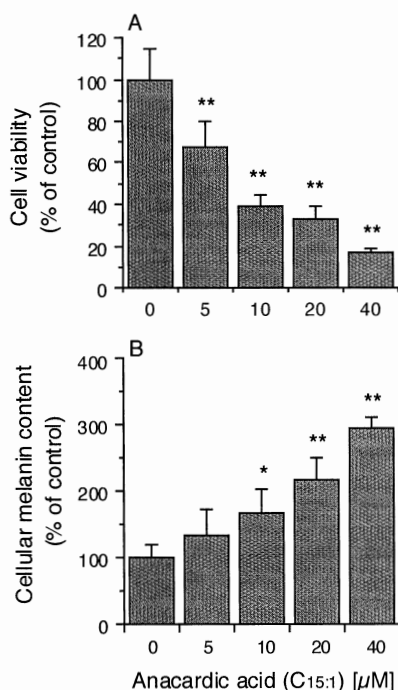


Fig. 6. (A) Viabilities of B16 melanoma cells following treatment with anacardic acid (C_{15:1}) for 72 h measured as described in 'Materials and Methods'; data are expressed as percentage of the number of viable cells observed with the control, and each column represents the mean \pm S.D. of at least 6 determinations. (B) Cellular melanin content in B16 melanoma cells following treatment with anacardic acid (C_{15:1}) for 72 h measured as percentage of melanin content per cell observed with the control, and each column represents the mean \pm S.D. 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.

toxicity with an IC₅₀ value of 50 μM (6.1 μg/ml), but neither salicyl alcohol (**5**) nor methyl salicylate (**6**) showed noticeable cytotoxicity up to 1 mM as judged by microscopic observation. The results observed suggest that optimization is achievable through the synthetic approach in which the molecular lipophilicity has a critical impact on the ability.

The knowledge obtained is applicable not only to achieve synthetic optimization but also to select appropriate natural products. The following is an example of the latter case based on this concept. Anacardic acids, isolated from the cashew *Anacardium occidentale* (Anacardiaceae), are salicylic acid derivatives with a nonisoprenoid C₁₅-alk-

(en)yl side chain. The addition of a C₁₅-alk(en)yl side chain to salicylic acid converts it to anacardic acids, which are amphipathic molecules, and therefore their hydrophobic properties dominate the properties of the molecules. Hence, anacardic acids are expected to act as membrane-active agents (surfactants). For example, the effects of 6[8(*Z*)-pentadecenyl]salicylic acid (**8**), which is referred to anacardic acid (C_{15:1}) for simplicity, on melanin formation in the same melanoma cells were examined. Anacardic acid (C_{15:1}) did not inhibit melanin production in cultured melanoma cells but rather enhanced it in a dose-dependent manner. The IC₅₀ value was established as 8 μM (2.8 μg/ml) and the number of viable cells at 40 μM was less than 20% of the control (Fig. 6A). The cell viability observed in the range of 5–40 μM was significantly different (*p* < 0.01) from the control. The melanin content per cell was shown to increase in a concentration-dependent manner up to 40 μM (Fig. 6B). The cellular melanin production observed in the range of 20–40 μM was significantly different (*p* < 0.01) from the control. It should be noted, however, that the melanin content per cell increased in a dose-dependent manner but the total amount of melanin was not exceeding the control.

Discussion

As long as a free carboxy group exists in the aromatic ring, hydroxyphenolic acids such as *para*- and *meta*-hydroxybenzoic acid bind to the binuclear copper-active center presumably with the more acidic carboxy group (Conrad *et al.*, 1994). These hydroxyphenolic acids are coordinated to one copper atom as monophenol substrates and positioned over the binuclear active site, and inhibit tyrosinase activity competitively (Wilcox *et al.*, 1985). However, this is unlikely the case for *ortho*-hydroxybenzoic acid and its derivatives. Substitution of the *ortho* position with a hydroxy group may prevent such binding. More importantly, the hydroxy and carboxy groups in these molecules form a quasi six-membered ring through intramolecular hydrogen bonding and as a result produce a stable chelate structure. Hence, the carboxy group of salicylic acid no longer binds as a substrate analogue. The chelated structure is known to stabilize as a complex with certain divalent metals. It seems that salicylic acid and its derivatives inhibit the enzyme, at least in part, by

their ability to chelate copper in the enzyme. However, salicylic acid is unlikely able to form a stable chelate complex since it does not possess a hydrophobic alkyl side chain in the molecule (Wilcox *et al.*, 1985; Nagabhushana *et al.*, 1995). The alternative possibility, that salicylic acid disrupts the tertiary structure of the enzyme through intermolecular hydrogen bonding, is more likely and reduces the affinity of the substrates to the enzyme. The conclusive interpretation remains to be clarified since the structure of tyrosinase used for this study has not yet been established. In either case, tyrosinase inhibitory activity in cell-free experiments is not correlated with cellular melanin formation, which is not simply to inhibit tyrosinase but involves more complex biochemical reactions.

Salicylic acid is a rare tyrosinase inhibitory phenolic acid in cell-free experiments. There are significant differences in the inhibition mechanisms of anacardic acids and salicylic acid as tyrosinase

inhibitors. The hydrophobic pentadecenyl side chain in anacardic acids plays an important role. However, the hydrophobic moiety alone is not enough to elicit the activity since cardanols, which possess the same side chain as anacardic acids, acted neither as substrates nor inhibitors (Kubo *et al.*, 1994). The hydrophilic head portion of anacardic acids first chelate the copper atom in the active site of the molecule, and then the hydrophobic tail portion begins to interact with the hydrophobic domain near the binuclear active site. The relevance of the results of *in vitro* experiments in simplified systems concerning the cellular activity needs to be carefully considered.

Acknowledgements

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