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Effects of Thymol on Mushroom Tyrosinase-Catalyzed Melanin Formation

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ABSTRACT: The novel inhibitory mechanism of thymol (2-isopropyl-5-methylphenol) on dopachrome formation by mushroom tyrosinase (EC 1.14.18.1) was identified. The UV−vis spectrum and oxygen consumption assays showed dopachrome formation using L-tyrosine as a substrate was suppressed by thymol. This inhibitory activity was reversed by the addition of a well-known radical scavenger, butylated hydroxyanisole (BHA). Further investigations using *N*-acetyl-L-tyrosine as a substrate with HPLC analysis suggested that thymol inhibits chemical redox reactions between dopaquinone and leukodopachrome instead of enzymatic reaction. This redox inhibitory activity of thymol was examined by using a model redox reaction with L-dihydroxyphenylalanine (L-DOPA) and *p*-benzoquinone. Thymol successfully inhibited oxidation of L-DOPA to dopaquinone, coupled with reduction of *p*-benzoquinone. Hence, the suppression of dopachrome formation by thymol is due to the inhibition of conversion of leukodopachrome to dopachrome. The antioxidant property of thymol is a key characteristic for the inhibitory mechanism of melanin synthesis.

KEYWORDS: melanin formation, tyrosinase, thymol, redox inhibitor

INTRODUCTION

Tyrosinase is the key enzyme in melanin synthesis. This copper-contained multifunctional oxidase catalyzes the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and further oxidizes L-DOPA to dopaquinone.[1] The reaction from dopaquinone to dopachrome proceeds nonenzymatically. The relatively stable intermediate compound, dopachrome, then undergoes chemical and enzymatic processes to form a biopolymer, brown/black melanin.[2,3] In various living systems, melanin is responsible for pigmentation and other functions such as chelation. However, undesirable browning is a considerable problem in the food industry or for cosmetic users. Furthermore, toxic oxidized products are commonly generated in the melanin synthetic pathway; for instance, high lipid content fruits such as avocado develop toxic oxide products in the browning process.[4] Thus, antibrowning reagents are frequently used. Previously, the most widespread method for antibrowning was the use of sulfiting agents,[5] but the U.S. Food and Drug Administration has banned sulfate agents for fruits and vegetables.[6] Hence, immediate finding of replacement of antibrowning reagents is essential. For these 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 because it is chemically oxidized to a nonfunctional form, dehydroascorbic acid.[7] These problems prompt us to search for safer and more stable melanin formation inhibitors from natural sources.

Thymol (**1**) and carvacrol (**2**) (see the structures in Figure 1) are naturally occurring monoterpene phenols that are the main constituents of thyme, an herb of the genus *Thymus*, and of origanum oils. Besides their odoriferous functions, thymol and carvacrol show antimicrobial activities.[8−10] Thus, thymol and carvacrol are commonly used as meat preservatives or flavorings in the food industry. In addition to their broad antimicrobial activities, the antioxidant activity of thyme essential oils was previously reported, and it has been known to be due to the presence of thymol and carvacrol.[11−13] As antioxidants, thymol and carvacrol protect food qualities and organisms from damage induced by oxidative stress. In contrast to these well-studied biological effects of thymol and carvacrol, their inhibitory actions on tyrosinase and melanin biosynthesis are poorly understood. The antibrowning effect in addition to the antimicrobial and antioxidant activities of thymol and carvacrol would help them to be considered as multifunctional additives. Hence, thymol was studied as a melanin formation inhibitor.

MATERIALS AND METHODS

Materials. Thyme oil, manufactured by NOW Foods (Bloomingdale, IL), was purchased at a local grocery store. Thymol, carvacrol, and arbutin were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine, L-DOPA, butylated hydroxyanisole (BHA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). 4-*tert*-Butylcatechol was purchased from Fluka Chemika (Buchs, Switzerland). 1-Methyl-3-methoxy-4-isopropylbenzene was purchased from SASF Supply Solutions (St. Louis, MO). *N*-Acetyl-L-tyrosine was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan).

Instrumentation: Gas Chromatography−Mass Spectrometry (GC-MS). The composition of thyme oil was analyzed by using a GC-MS system (GC-17A/QP5050; Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific Inc.). The temperature program was as follows: 45 °C for 8 min, followed by increases of 2.5 °C/min to 180 °C and 10 °C/min to 250 °C, and holding for 3 min. The other parameters were

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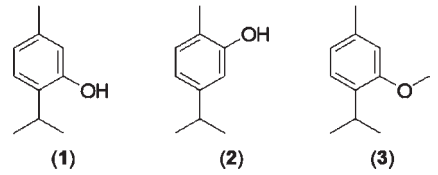


Figure 1. Chemical structures of thymol and its related compounds.

as follows: injection temperature, 250 °C; ion source temperature, 250 °C; ionization energy, 70 eV; carrier gas, helium (He) at 1.7 mL/min; injection volume, 1 μL (90 μg/mL Et_2O); split ratio, 1:20; mass range, m/z 50−450.

Enzyme/Spectrophotometric Assay. General procedures were the same as in previous works [14,15] but slightly modified. All assays were performed in triplicate on separate occasions. 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.[16] The current experiment used 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 general, L-tyrosine was mainly used as a substrate because it is a natural substrate of tyrosinase. 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. Results were expressed in absorbance units with appropriate wavelength (nm), and all data were processed with SigmaPlot 10 software (Systat Software Inc.).

Oxygen Consumption Assay. The general procedure was previously described.[14,15] Briefly, 100 μL of a 3 mM L-DOPA or L-tyrosine aqueous solution was mixed with 2.1 mL of distilled H_2O and 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-jacketed chamber 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 micromolar, and calibration of an oxygen electrode was performed by using 4-*tert*-butylcatechol and excess tyrosinase according to a previous paper.[17] All assays were performed in triplicate on separate occasions.

HPLC Analysis. Time-dependent consumption of substrates and 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.) and a 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 the 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. On 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.

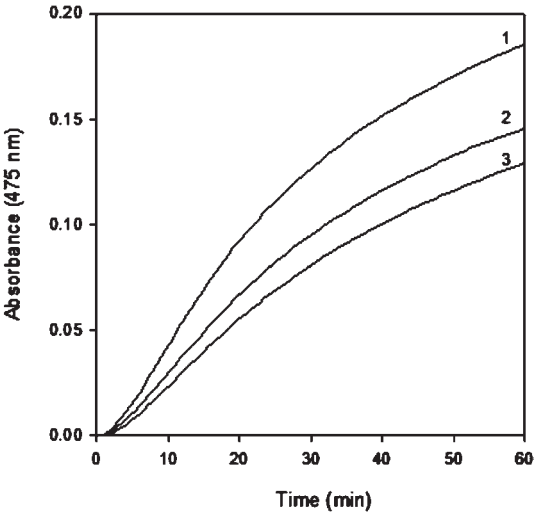


Figure 2. UV−vis spectra at 475 nm obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the presence or absence of thyme oil for 60 min. Concentrations of thyme oil were selected at 33.3 μg/mL (2) and 66.6 μg/mL (3). Line 1 represents oxidation of L-tyrosine by mushroom tyrosinase in the absence of thyme oil.

RESULTS AND DISCUSSION

The investigation began with thyme oil because it is commercially available and also found in food supplies. Thyme oil exhibited a concentration-dependent inhibitory effect on the tyrosinase-catalyzed oxidation of L-tyrosine with 60 min of reaction time (Figure 2), whereas there was no inhibition effect against the L-DOPA oxidation (data not shown). As the concentration of thyme oil increased, the formation of dopachrome decreased. Due to the solubility problem, the highest tested concentration of the oil was 66.6 μg/mL, and about 35% of dopachrome formation was suppressed at this concentration. Typical lag phases (observed for the hydroxylation of L-tyrosine) were observed in all of the curves (Figure 2). However, the extension of the lag phase, which is commonly observed with the effect of monophenol substrate analogues, was not observed with addition of thyme oil. The components of thyme oil were characterized by using a GC-MS system. The major component of thyme oil was thymol (75.71%), and about 80% of contents were thymol and carvacrol in thyme oil (data not shown). As described above, thymol and carvacrol are the bioactive compounds in thyme oil for various biological activities. Hence, we concluded that, in thyme oil, thymol and carvacrol are the major bioactive compounds for melanogenesis inhibition.

As well as thyme essential oil inhibits dopachrome formation, thymol exhibited a concentration-dependent inhibitory effect on the dopachrome formation (Figure 3A). At 475 nm, about 25% of dopachrome formation was suppressed with 400 μM thymol after 60 min of incubation. The consumption of oxygen was also inhibited dose-dependently with the addition of thymol (Figure 3B). No change in lag phase was observed for any curves in both UV and oxygen consumption assays (Figure 3). However, the inhibitory effect was not observed with L-DOPA in

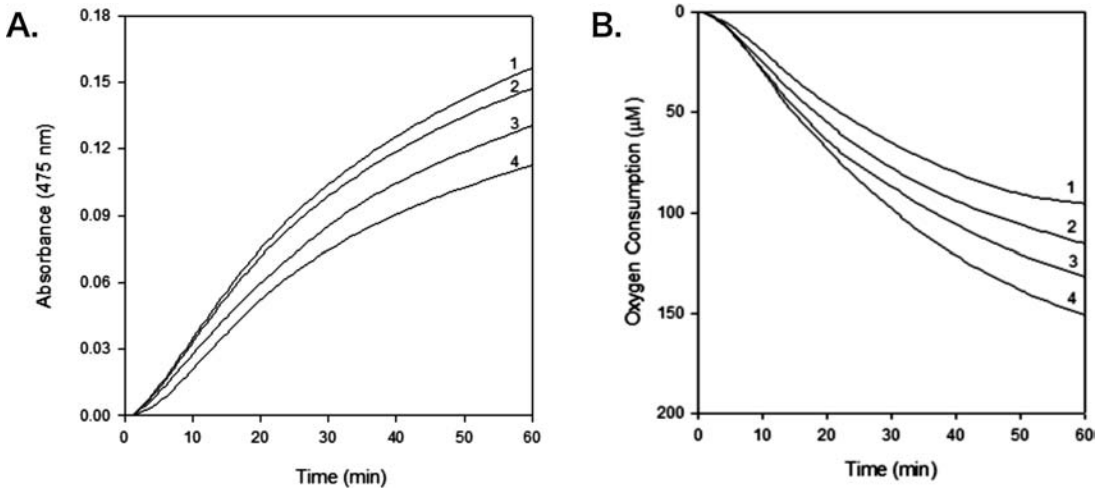


Figure 3. (A) UV−vis spectra at 475 nm obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the presence or absence of thymol for 60 min. Concentrations of thymol were selected at 100 μM (2), 200 μM (3), and 400 μM (4). Line 1 represents oxidation of L-tyrosine by mushroom tyrosinase in the absence of thymol. (B) Oxygen consumption of oxidation of L-tyrosine (100 μM) by mushroom tyrosinase in the presence or absence of thymol for 60 min. The concentrations of thymol were 400 μM (1), 200 μM (3), and 100 μM (3). Line 4 represents the oxygen consumption of oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the absence of thymol.

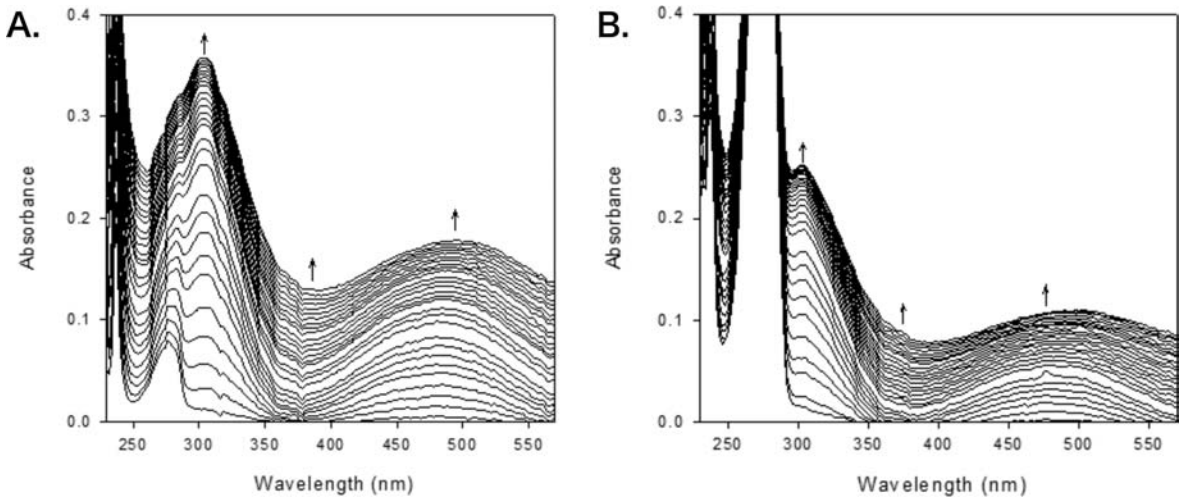


Figure 4. Consecutive UV−vis spectra obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the absence (A) or presence (B) of 400 μM thymol for 60 min. Scan speed was at 2 min intervals for 30 s. The arrows (↑) designate the evolution of the peak.

UV−vis and oxygen consumption assays (data not shown). Similar inhibitory effects of thymol were observed using carvacrol in both spectrophotometric and oxygen consumption assays (data not shown). Interestingly, this inhibitory effect on dopachrome synthesis (475 nm) and oxygen consumption with L-tyrosine was not observed when thymol methyl ether (1-methyl-3-methoxy-4-isopropylbenzene) (**3**) was used (data not shown). Blocking the phenolic OH of thymol with a methyl group diminished the inhibitory activity that is observed with thymol. This structure−activity relationship (SAR) study suggests that the presence of the phenolic hydroxyl group of thymol or carvacrol is essential for the inhibition.

The consecutive UV−vis spectrum of L-tyrosine oxidation by tyrosinase in the presence of 400 μM thymol was obtained. The evolution of the peak at 475 nm, corresponding to dopachrome formation, decreased with the addition of thymol (Figure 4). About 40% of dopachrome formation was suppressed in the spectrophotometric assay. Furthermore, at 320 nm, the peak developed in both spectra, but there was less increase in Figure 4B. The evolution of the peak at this wavelength refers to the formation of a quinone product, dopaquinone, in the melanin synthesis process. Basically, the same result was observed in both spectrophotometric assays at 475 nm and the consecutive UV−vis assay.

The effect of thymol on the E_{oxy} state of tyrosinase was examined by the addition of 10 μM L-DOPA as a cofactor. Introduction of L-DOPA to tyrosinase reduces its state from E_{met} to E_{oxy}, which are resting and active form of tyrosinase, respectively. L-DOPA was preincubated with tyrosinase for 3 min to convert all of the enzyme to E_{oxy}, and then dopachrome formation was measured after the addition of 100 μM L-tyrosine with or without 400 μM thymol. Addition of a cofactor did not show any effect on the inhibition of dopachrome formation (Figure 5) compared to the measurement result without a cofactor (Figure 3). Furthermore, previous results

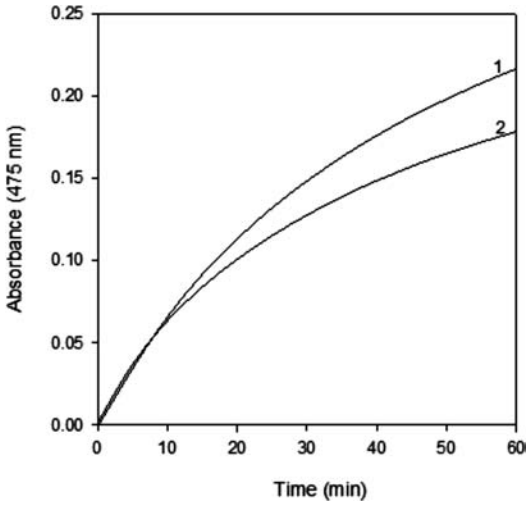


Figure 5. UV−vis spectra at 475 nm obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the presence or absence of thymol and a cofactor for 60 min. Mushroom tyrosinase was preincubated with 10 μM L-DOPA for 3 min, and then L-tyrosine was added. Concentrations of thymol were selected at 400 μM (2). Line 1 represents oxidation of L-tyrosine by mushroom tyrosinase in the absence of thymol.

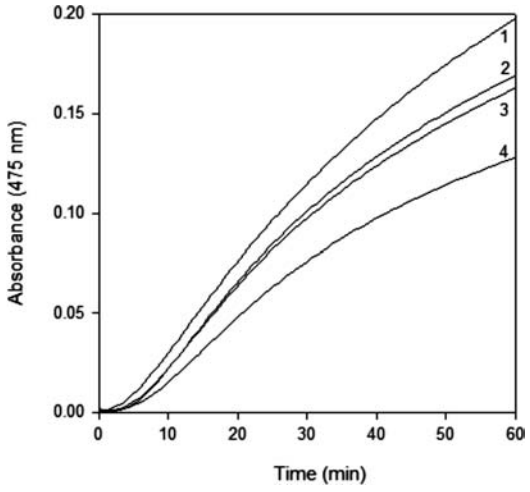


Figure 6. UV−vis spectra at 475 nm obtained in the oxidation of 100 μM L-tyrosine by mushroom tyrosinase in the presence or absence of thymol and butylated hydroxyanisole mixture for 60 min. Concentration of thymol was 100 μM. Concentrations of butylated hydroxyanisole were selected at 100 μM (2), 50 μM (3), and 0 μM (4). Line 1 represents oxidation of L-tyrosine by mushroom tyrosinase in the absence of both thymol and butylated hydroxyanisole.

showing that thymol (and thyme oil) did not affect the lag phase support this result. Thus, these suggest that thymol does not interact with the E_{oxy} state of tyrosinase, and it can be concluded that thymol does not act as a monophenol analogue.

Surprisingly, the inhibitory effect of thymol was masked with the application of a radical scavenger, BHA. As the concentration of BHA was increased to 100 μM, the inhibitory effect due to thymol on dopachrome formation was dose-dependently decreased (Figure 6). With 100 μM BHA and thymol, about 20% of the inhibitory activity on dopachrome formation was suppressed. This suggests that BHA affected the antimelanogenic action of

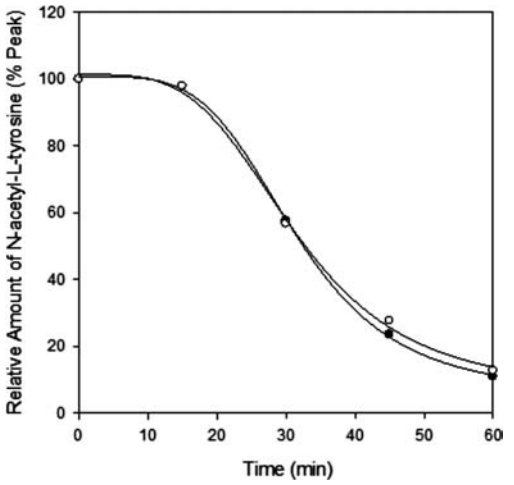


Figure 7. HPLC analysis of *N*-acetyl-L-tyrosine (100 μM) oxidation by tyrosinase in the presence (○) or absence (●) of thymol (400 μM). Sampling time was chosen at 0, 15, 30, 45, and 60 min. HPLC operating conditions were as follows: Develosil ODS-UG-5 (Nomura Chemical, Co., Ltd., Seto-Shi, Aichi, Japan); solvent, 15% 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. Curve fitting to smoothly connect the points is done with SigmaPlot (Systat Software, Inc.).

thymol; in other words, the phenoxy radical formation and proton donation of thymol are linked to the antimelanogenic activity of thymol. On the other hand, the inhibitory activity was enhanced when tyrosinase was preincubated with thymol for 10 min (data not shown). In this treatment, preincubation gives extra time for the proton to dissociate from the hydroxyl group of thymol to form a phenoxy radical. Thus, the antioxidant effect of thymol is key for inhibition of dopachrome formation.

The involvement of thymol in enzymatic reactions in melanin synthesis was investigated with *N*-acetyl-L-tyrosine. Using *N*-acetyl-L-tyrosine as a substrate, tyrosinase oxidizes substrate to synthesize to *N*-acetyldopaquinone. Because of the presence of an acetate group on the *N*-group of an amino acid, intracyclization of dopaquinone to leukodopachrome is blocked. Hence, only the tyrosinase-catalyzed reaction step in melanin synthesis process can be examined using this compound as a substrate. When *N*-acetyl-L-tyrosine is applied to tyrosinase in the presence of 400 μM thymol, the consumption of *N*-acetyl-L-tyrosine was no different from the control (Figure 7). This explains why thymol did not show any involvement in the enzymatic reactions. Thus, it appears that thymol is not a "tyrosinase" inhibitor.

The formation of dopachrome from dopaquinone is extremely rapid. Therefore, an alternative model of this reaction is required to test whether thymol affects this nonenzymatic process. L-DOPA and *p*-benzoquinone were used as models instead of leukodopachrome and dopaquinone, respectively. The consumption of *p*-benzoquinone was measured by using HPLC analysis. As thymol was incubated in this model system, the consumption of *p*-benzoquinone was decreased about 20% in 10 min (Figure 8, top). At the same time, the formation of hydroquinone was also measured in the same manner. About 20% of hydroquinone formation was suppressed with the addition of 400 μM thymol (Figure 8, bottom). These results suggest thymol quenched the redox reaction between L-DOPA and *p*-benzoquinone (Scheme 1). On the basis of these results, it is logical to assume that the inhibitory action of thymol on dopachrome formation is due to the

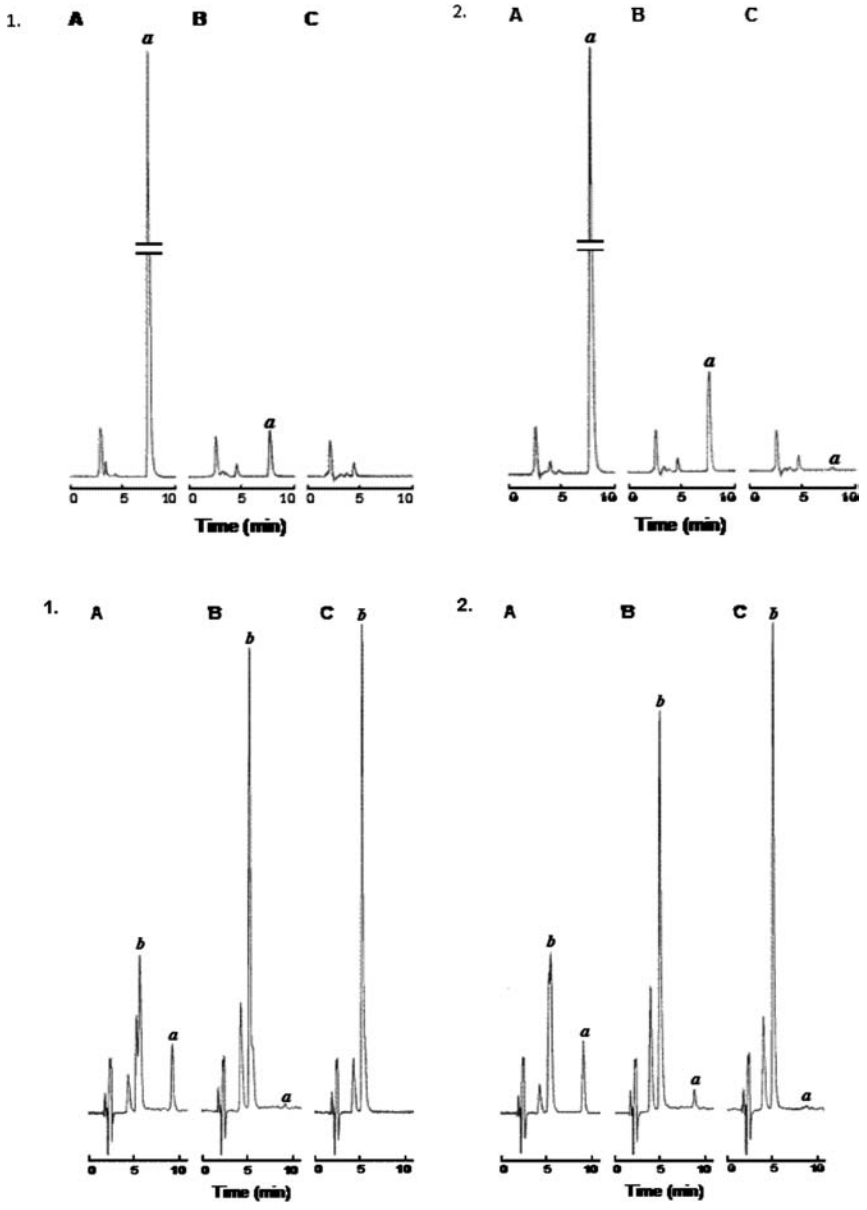


Figure 8. (Top) HPLC analysis of the redox reaction of *p*-benzoquinone (400 μM) and L-DOPA (200 M) in the absence (1) or presence (2) of 400 μM thymol in buffer (without tyrosinase). Sampling time was chosen at 0 min (A), 10 min (B), and 20 min (C). HPLC operating conditions were as follows: Develosil ODS-UG-5 (Nomura Chemical, Cp., Ltd., Seto-Shi, Aichi, Japan); solvent, 10% MeCN/H_2O containing 0.2% TFA; flow rate, 1.0 mL/min; detection, UV at 245 nm, 0.02 range; injected amount, 25 μL. Peak *a* represents *p*-benzoquinone. (Bottom) HPLC analysis of L-DOPA (200 μM) oxidation by *p*-benzoquinone in the absence (1) or presence (2) of 400 μM thymol in buffer (without tyrosinase). Sampling time was chosen at 0 min (A), 10 min (B), and 20 min (C). 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, 0.04 range; injected amount, 25 μL. Peaks *a* and *b* represent *p*-benzoquinone and hydroquinone, respectively.

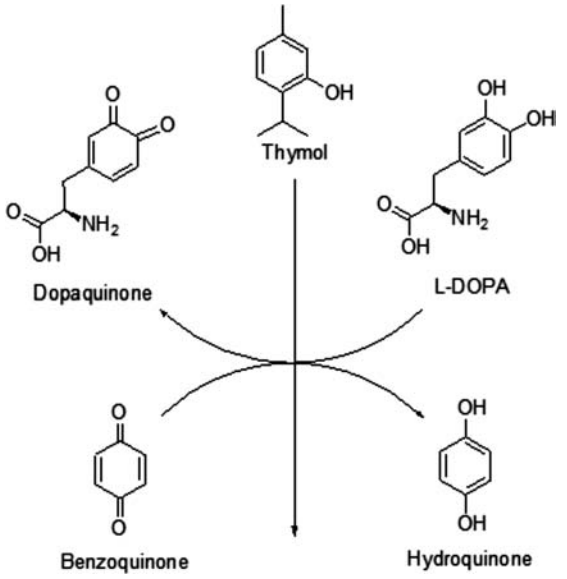
suppression of conversion of leukodopachrome to dopachrome (Scheme 2).

Antioxidants act in a variety of ways, including direct quenching of reactive oxygen species, inhibition of enzymes involved in the production of the reactive oxygen species, chelation of low-valent metal ions such as Fe^{2+} or Cu^{2+}, and regeneration of membrane-bound antioxidants such as α-tocopherol.[18] On the basis of the data obtained, it may be logical to assume that thymol inhibits the redox reaction in melanin synthetic pathway. It seems that this inhibition of melanin formation is due to the radical scavenging activity of thymol. Direct proton donation from thymol interrupts electron flow between dopaquinone and leukodopachrome. The electron-donating isopropyl and methyl groups in the ortho position contribute to the stability of the phenoxy radical and their antioxidative activities, similar to previous results.[19,20] Furthermore, various monophenol compounds are recognized by tyrosinase and sometimes act as monophenol substrate analogues.[21,22] However, steric hindrance due to methyl or isopropyl groups adjacent to hydroxyl groups prevents tyrosinase from recognizing thymol as a monophenol. Thus, thymol is considered to obe a unique melanin synthesis inhibitor, but not an enzyme inhibitor.

Excessive melanogenesis generates free radicals and initiates unwanted radical reactions such as lipid peroxidation.[23] Subsequently, oxidations turn active intracellular chemicals into nonactive forms; for instance, vitamin C turns into dehydroascorbic acid. Toxicity due to oxidized products also causes serious damage, such as diabetes mellitus and coronary arteriosclerosis,[24,25] as well as being linked with aging and carcinogenesis.[26] As described, the antimelanogenic activity of thymol is linked with its antioxidant effect; in fact, the melanin formation inhibitor thymol can help protect human health against oxidative damage as well as the prevent undesirable browning.

Safety is a primary concern for food or cosmetic additives. The radical-scavenging effect of thymol depletes an active electron in melanin synthesis, but antioxidant-related phenoxy radicals are newly formed; the fate of radicals newly formed through the antioxidant effect in humans is still unclear. Because of this property, antioxidants are also known as a double-edged sword. However, in the case of thymol, useful antioxidant properties have been reported without prooxidant effects.[11] From our experimental results, the inhibitory mechanism of thymol on melanogenesis is due to the inhibition of the redox reaction between dopaquinone and leukodopachrome without any interaction with tyrosinase. Thus, this antimelanogenic effect (directly link to the antioxidative effect) in addition to antimicrobial and antioxidant activities allows thymol to be a multifunctional additive. Furthermore, thymol is extracted from food spices and, hence, it may be an alternate choice to non-natural food additives for the purpose of antibrowning. Despite its advantage as a food additive, the biological functions, including the mechanism of toxicity, are still under investigation. Further investigation is required to clarify this problem before thymol can be used as an antibrowning agent in the food industry.

Scheme 1. Inhibitory Effect of Thymol on the Redox Reaction between *p*-Benzoquinone and L-DOPA



DISCLOSURE

This work was accepted in part at the General Paper Session of the Division of Agricultural and Food Chemistry for the 241st ACS National Meeting in Anaheim, CA.

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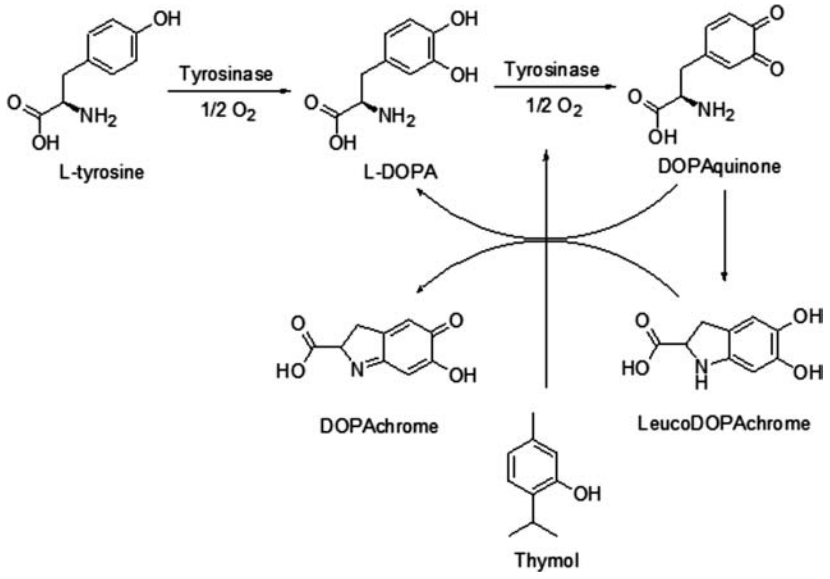
Scheme 2. Proposed Scheme of Inhibitory Mechanism of Melanin Formation by Thymol



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