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Identification of Oxidation Product of Arbutin in Mushroom Tyrosinase Assay System

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Abstract—In order to elucidate whitening mechanisms of arbutin (hydroquinone-*O*-β-D-glucopyranoside), its effects on mushroom tyrosinase were analyzed by spectrophotometric, polarographic, and HPLC experiments. It was found that as soon as catalytic amounts of L-DOPA become available as a cofactor, arbutin acts as a monophenol substrate. A significant enzymatic product was identified as 3,4-dihydroxyphenyl-*O*-β-D-glucopyranoside by NMR and MS experiments.

Tyrosinase (EC 1.14.18.1), a copper containing enzyme, catalyzes two distinct reactions of melanin synthesis,[1] the hydroxylation of a monophenol (monophenolase) and the conversion of an *o*-diphenol to the corresponding *o*-quinone (*o*-diphenolase). The enzymatic oxidation of L-tyrosine to melanin is of considerable importance since melanin has many functions, including light absorption and scattering. Alterations in melanin synthesis occur in many disease states. Melanin pigments are also found in the mammalian brain. Tyrosinase may play a role in neuromelanin formation in the human brain, particularly in substantia nigra, and could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease.[2] Melanoma specific anticarcinogenic activity is known to be linked with tyrosinase activity.[3] Tyrosinase inhibitors have become increasingly important in medicinal[4] and cosmetic products,[5] primarily in relation to hyperpigmentation.

Arbutin, hydroquinone-*O*-β-D-glucopyranoside (**1**), has been used as a whitening agent in cosmetics.[6] This hydroquinone glucoside was previously reported to show a dose-dependent inhibitory effect on the oxidation of L-DOPA catalyzed by mushroom tyrosinase with an IC_{50} of 8.4 mM.[7] This inhibition analyzed by Lineweaver–Burk plots indicate arbutin to be a competitive inhibitor.[6] However, the IC_{50} value reported is extremely low compared to that of kojic acid (280 μM, approximately)[8] which has also been used in cosmetics.[9] Despite arbutin's wide use as a whitening agent, its depigmenting mechanism on a molecular basis is not yet fully understood. This prompted us further study of arbutin to gain new insights into the regulatory control of human melanogenesis.

Dose-dependent experiments were performed on mushroom tyrosinase by L-DOPA as a substrate for estimating arbutin's IC_{50} value.[10] The assay was carried out in an air-saturated aqueous solution at 30 °C. Tyrosinase catalyzes a reaction between two substrates, a phenolic compound (L-DOPA) and oxygen. Hence, the reaction can be determined by spectrophotometry (dopachrome formation) and/or by polarography (oxygen consumption). The concentration of arbutin was selected up to 32 mM which was about 4-fold of the reported IC_{50} value.[7] In spectrophotomeric experiments, arbutin was inhibitory on the enzyme activity in a dose-dependent manner. However, the IC_{50} value could not be estimated less than 32 mM in our experimental conditions. The rate of initial oxygen consumption was not dependent on arbutin concentrations up to 32 mM in polarographic experiments and hence, obtaining IC_{50} value was difficult within this concentration.[11] Thus, increasing the concentration of arbutin did not affect oxygen consumption but slightly affected dopachrome formation. These results were well contradictory with a previous report[7] but indicated that the oxygen in the cuvet was consumed for other than the oxidation of L-DOPA.

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Commercial mushroom tyrosinase is known to contain mainly the *met*-form, which can oxidize an *o*-diphenol to the corresponding *o*-quinone while *oxy*-tyrosinase oxidizes both monophenol and *o*-diphenol.[12] However, if *o*-diphenol compounds emerge as a cofactor in the reaction medium, *met*-tyrosinase can be quickly converted to the *oxy*-form.[13] This phenomenon occurred in the very early stage of the above dose-dependent experiments, which is possibly one of the reasons for their interesting results. As a result, pre-incubation and cofactor addition experiments were performed to estimate the effect of *oxy*-tyrosinase on arbutin as a monophenol.

Slight oxygen consumption was observed in the incubated experiment with tyrosinase and arbutin (0.5 or 8.0 mM) for 20 min (Fig. 1). As soon as a catalytic amount of L-DOPA (10 μM) was added into the incubated mixture, oxygen concentration in the cuvet rapidly decreased. After 15 min from the time cofactor was added, oxygen consumption using 8.0 or 0.5 mM of arbutin reached about 100 or 56 μM, respectively.

Incubated experiments with the enzyme and 0.5 mM of arbutin led to the observation of insignificant changes on its UV spectra within 20 min (data not shown). Treating 10 μM of L-DOPA in 5 min converted the original spectrum into a complex figure (Fig. 2). Thus, the cofactor effect of L-DOPA was clearly shown. The changes mainly occurred in increasing absorbance at 286, 320, and 460 nm, which were similar to the result on L-DOPA oxidation by tyrosinase. However, the resulting peaks were higher than those compared to the peaks forming by the enzymatic reaction carried out 10 μM of L-DOPA as a substrate without arbutin. In order to further investigate tyrosinase effects on arbutin, analytical experiments of the reaction medium were performed by HPLC.

Chromatographic analyses in the tyrosinase reaction on 0.5 mM of arbutin with 10 min intervals, showed peak **a** (t_R 7.5 min), identified as arbutin, was slightly diminished while an unknown peak **b** (t_R 6.6 min) appeared (Fig. 3). Interestingly, adding a catalytic amount of L-DOPA hastened to change both peaks that were looked up as Figure 4. These results led to an understanding that arbutin acted as a monophenol substrate by *oxy*-tyrosinase and peak **b** represented one of the products by enzymatic reaction.

The retention time of peak **b** was earlier than that of peak **a** on chromatograms. In general, *o*-quinone, a possible product by tyrosinase, should be eluted later than *o*-diphenol by HPLC analysis.[14] This questionable observation of peak **b** prompted further investigation of tyrosinase catalytic mechanism on arbutin. The identification procedure was executed in the presence of 1.0 mM of L-ascorbic acid in the reaction medium. This effect is to reduce the *o*-quinone back to the corresponding *o*-diphenol, and L-ascorbic acid can also be a cofactor on *met*-tyrosinase.[15]

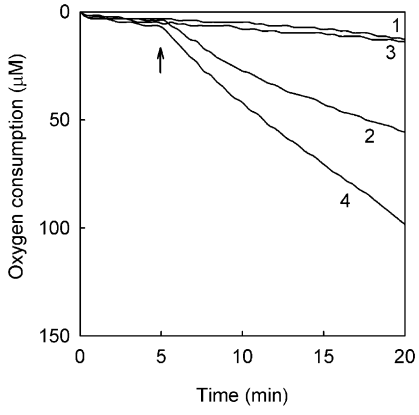


Figure 1. Oxygen consumption of arbutin in the presence of mushroom tyrosinase. The concentrations were selected at 0.5 mM (1, 2) or 8.0 mM (3, 4). The arrow (↑) indicated the time when catalytic amount (10 μM) of L-DOPA was added (2, 4).

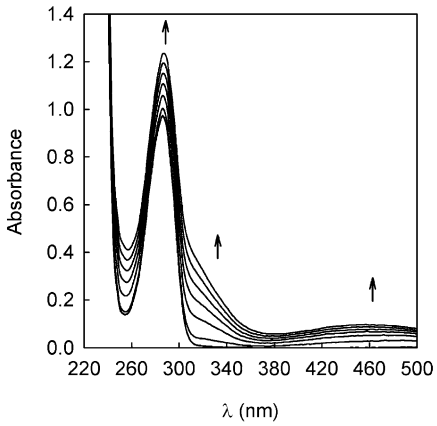


Figure 2. Consecutive spectra obtained in the oxidation of 0.5 mM of arbutin by mushroom tyrosinase for 20 min. The catalytic amount of L-DOPA (10 μM) was added at 5 min. Scan speed was at 2-min intervals for 30 s. The arrows (↑) designate the evolution of the peak.

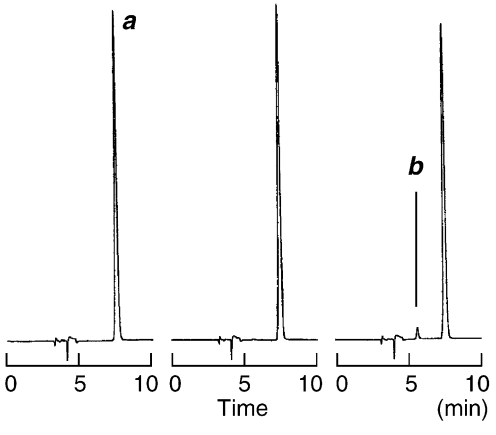


Figure 3. HPLC analysis of the reaction medium with 0.5 mM of arbutin and tyrosinase. Sampling time was chosen at 0 min (left), 10 min (middle), and 20 min (right), respectively. The HPLC operating conditions were as follows: column; Capcell Pak C-18 (Shiseido, Tokyo, Japan), solvent; 2.5% MeCN/H_2O containing 0.1% TFA, flow rate; 0.8 mL/min, detection; UV at 280 nm, injected amount; 20 μL from 3-mL assay system.

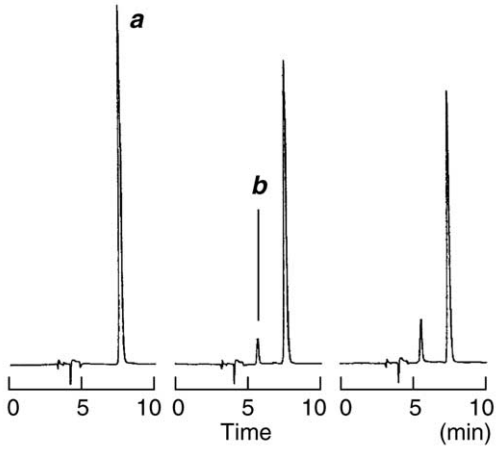


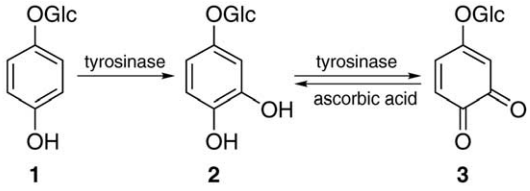
Figure 4. HPLC analysis of the reaction medium with 0.5 mM of arbutin and tyrosinase. The catalytic amount of L-DOPA (10 μM) was added at 5 min. Sampling time was chosen at 0 min (left), 10 min (middle), and 20 min (right), respectively. The HPLC operating conditions were same as Figure 3.

In the spectrophotometric observation, the materials with maximam absorbance at 320 and 460 nm which indicate quinones and polymeric compounds, were not detected. Chromatograms obtained under those conditions indicated that peak **b** was increased for 20 min, time-dependently (data not shown). Although L-ascorbic acid (5.0 mM) caused 50% inactivation of mushroom tyrosinase after 130 min with addition of enzyme,[16] a 10 times large-scale reaction being stirred overnight, followed by preparative HPLC, gave 3,4-dihydroxyphenyl-*O*-β-D-glucopyranoside (**2**) as a major enzymatic product in 29% yield.[17] Consequently, peak **b** was identified as **2** by further HPLC analysis.

Pre-incubated enzyme is mostly *met*-tyrosinase, which is known as the resting form. Mainly, the inhibition activity exerted by arbutin could be based on the assumption that arbutin binds as a monophenol substrate analogue to the *met*-form of tyrosinase. The resulting complex is inactive and is also known as the dead-end pathway.[18] Extended lag times could be observed by spectrophotometry in the experiment using L-tyrosine as a substrate. It should be noted that commercial tyrosinase is known to contain a small amount of the *oxy*-form. Under this condition, arbutin can be greatly degraded by the enzyme.

Reaction mechanism of tyrosinase was well presented via the results of detailed biophysical and enzymological studies.[12,19] There are two current points of view on tyrosinase catalytic mechanism of the release procedure of products from enzyme using monophenol substrate: one is that the enzyme can only release *o*-quinone as a product[20] and the other view is that the enzyme can release both *o*-quinone and *o*-diphenol.[21] When arbutin was used as a substrate, this enzymatic reaction gave rise to the *o*-diphenol product (**2**) in the medium as well as *o*-quinone (**3**) (Scheme 1).

Although the inhibition of mushroom tyrosinase activity has recently been reported not to correlate with that



Scheme 1. Oxidation of arbutin (**1**) to corresponding compounds by mushroom tyrosinase.

of cellular tyrosinase and melanin production in human melanocytes,[6] the current results may provide a more precise depigmenting mechanism of arbutin on a molecular basis. The properties of arbutin acting as a substrate by tyrosinase were suggested in a previous report.[5] If arbutin is a substrate by the *oxy*-form of the human tyrosinase, it would be beneficial for arbutin to be reduced to the dopachrome formation by spectrophotometry. In addition, the enzyme would drastically consume intracellular oxygen as one of the substrates by tyrosinase. Presumably, they create gaps in the medicinal efficacy of arbutin between inhibition activity of the melanin formation and tyrosinase inhibitory activity. However, it is known to have an effect at the post-translational level since it did not significantly change in protein content, molecular size of tyrosinase, and its mRNA expression in human cells.[6,22,23]

In conclusion, we demonstrated that arbutin acts as a substrate by *oxy*-tyrosinase and the corresponding *o*-diphenol can be released from the enzyme. These results may indicate other clues on how arbutin acts as a whitening agent. Further investigations are possibly needed including the effects of digested products of arbutin and exhausted oxygen in human cells.

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