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原著論文
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Inhibition of Pigmentation due to a Copper-Containing Enzyme, Tyrosinase, by Oxalates and Aromatic Sulfinates

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Abstract

It has been revealed that pigmentation based on the oxidative polymerization of 3,4-dihydroxyphenylalanine (dopa) by the action of a copper-containing enzyme, tyrosinase, is suppressed by free oxalic acid and its salts (oxalates) as well as by sodium benzenesulfinate (NaBS) and sodium p-toluenesulfinate (NaTS). The results of this investigation on the inhibition mechanisms suggested that oxalates non-competitively inhibit the activity of tyrosinase due to the formation of a chelate linkage with the functional copper ions in the enzyme molecule and that the two aromatic sulfinates react with dopaquinone formed by the enzymatic oxidation of dopa to afford their stable derivatives which are no longer transformed and polymerized.

Key words : tyrosinase, pigmentation, inhibitor, oxalates, aromatic sulfinates.

Introduction

Tyrosinase (EC 1.14.18.1) is an enzyme, which contains a pair of copper ions (Cu^{++}) in the active site [1], that catalyzes the oxidative reaction of tyrosine (= 4-hydroxyphenylalanine) to produce 3,4-dihydroxyphenylalanine (dopa) and also that of dopa to generate the corresponding orthoquinone, dopaquinone (DQ). The formed DQ is then simultaneously transformed and polymerized to afford melanin-type pigments. Tyrosinase is widely distributed in the natural world including animal skin and plant tissues, and the elevation of its activity is often accompanied by a harmful and/or inconvenient pigmentation. Therefore, a variety of compounds, which can act as inhibitors of the enzyme activity and/or as antioxidants reducing DQ to dopa, have been surveyed and prepared for use in the cosmetic and medical fields, as well as in food protection technology, etc. [2]. As will be described later in detail, the author revealed that pigmentation by the action of tyrosinase on dopa as the substrates suppressed by free oxalic acid and its salts (oxalates), as well as by sodium benzenesulfinate (NaBS) and sodium p-toluenesulfinate (NaTS). This paper describes the inhibition mechanisms of the tyrosinase-induced pigmentation by oxalates and the two aromatic sulfinates.

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Materials and Methods

Materials: Mushroom tyrosinase was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oxalic acid and its salts of sodium (Na), potassium (K) and ammonium (NH_4), as well as NaBS and NaTS, were purchased from Kanto Chemicals.

Examinations of the enzyme inhibition: All the samples (oxalic acid, Na oxalate, K oxalate, NH_4 oxalate, NaBS and NaTS) were dissolved in deionized water (DW). For the enzyme assay, 1.0 ml each of 3.3 mM dopa (in DW) was mixed with 1.8 ml each of 100 mM phosphate buffer (pH 6.8), and then, 0.1 ml each of the sample solution with various concentrations up to 10 mM and 0.1 ml each of mushroom tyrosinase (120 units, in DW) were added. The reaction was started by the addition of the enzyme solution, and the initial rate of the linear increase in the optical density at 470 nm was measured at room temperature. These procedures are essentially the same as those reported by Lee [3] who identified some cinnamic acid derivatives as tyrosinase inhibitors in the extracts from the roots of *Pulsatilla cernua*. In

order to elucidate the inhibition mode by oxalates, kinetic treatments of the data were performed on the basis of varying the concentrations of the substrate (dopa) and the inhibitors (oxalates). The effects of the formation of a chelate linkage between the oxalates and the functional copper ions in the enzyme molecules were evaluated by the detection of an alleviated inhibition in the presence of copper sulfate ($CuSO_4$) with the final concentrations of up to 1.0 mM.

Analyses of the reaction products: In the assays for the detection of the effects by NaBS and NaTS, a constant volume of each reaction mixture with or without the samples was withdrawn every 30 sec for 3 min after the reaction started, then heated at 100 ℃ for 3 min to inactivate the enzyme, followed by centrifuging ultrafiltration with a Centricon-10 miniconcentrator to remove the enzyme protein. A constant volume each of the ultrafiltrates was then analyzed by the reversed-phase high-performance liquid chromatography (HPLC) under the conditions for the separation of dopa and its oxidized products [4]. The reaction products, which appeared only in the presence of NaBS and NaTS, were isolated by HPLC, and subjected to instrumental analyses employing fast atom boundary-mass spectroscopy (FAB-MS) and proton magnetic resonance spectroscopy (^{1}H-NMR), along with the authentic samples of dopa, NaBS and NaTS.

Results and Discussion

Under the employed conditions, oxalic acid inhibited the tyrosinase-induced pigmentation, and its concentration producing a 50% inhibition (IC_{50}) was ca. 5.0 mM. Varying the substrate (dopa) and the inhibitor (oxalic acid) concentrations did not change the Km value of the enzyme (ca. 0.27 mM), suggesting that the inhibition mode is the non-compeptitive type. The addition of Cu^{++} to the reaction mixtures concentration-dependently alleviated the inhibition. Indeed, in the presence of 1 mM Cu^{++}, the inhibition rate by 5.0 mM of oxalic acid was less than 10 %, indicating that more than 80% of the activity was recovered. All the examined salts of oxalic acid exhibited essentially the same results as above, although their IC_{50} values (ca. 6.5-9.0 mM) were slightly greater than that of the free oxalic acid. These results show that oxalates non-competitively inhibit the tyrosinase activity due to the formation of a chelate linkage with the functional copper ions in the enzyme. It has been known that tyrosinase is inhibited in a similar mode by chelating agents, such as ethylenediaminetetraacetate [5] and citric acid [6].

The pigmentation was also suppressed by NaBS and NaTS. Their IC_{50} values (2.0 mM and 1.5 mM, respectively) were lower than the oxalates during the first 2 min, although the inhibitory effects were time-dependently reduced and became weaker than the oxalates 10 min after the reaction was started. From the HPLC analysis of the reaction mixtures, the ratio of the time-dependent decrease in the concentration of dopa, the substrate, was not different between the samples with or without NaBS and NaTS, while two peaks responsible for compounds having absorbance at 280 nm (for aromatic ring) and longer retention times (for the more non-polar structure) than dopa, NaBS and

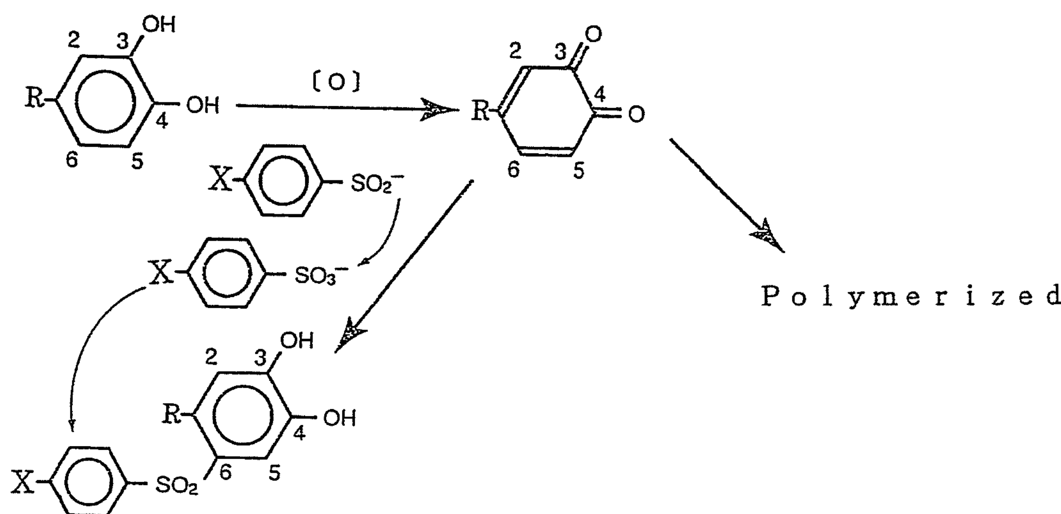

Fig. 1. Derivatization of dopaquinone (DQ) by benzenesulfinate and p-toluenesulfinate ions
R=OOC-CH(NH_3^+)CH_2, X=H: benzenesulfinate ion, X= CH_3: p-toluenesulfinate ion

NaTS, were observed only on the chromatograms for the samples containing NaBS and NaTS. They were isolated by HPLC and identified as 6-benzosulfonyldopa (=2-benzosulfonyl-4,5-dihydroxyphenylalanine) and 6-p-toluenesulfonyldopa (=2-p-toluenesulfonyl-4,5-dihydroxyphenylalanine) on the basis of the data of FAB-MS (the peaks for $M+1^+$ at m/z=338 for $C_{15}H_{15}O_6NS+1$ and 352 for $C_{16}H_{17}O_6NS+1$) and ^1H-NMR (appearance of signals at 7.4-8.1 ppm for protons for the benzosulfonyl residues with or without those for the methyl group at 2.4 ppm and the disappearance of signals at 6.7 ppm for a proton attaching to the aromatic C-6 of dopa), respectively. These data suggested that, as shown in Fig. 1, the benzenesulfinate ion ($C_6H_5SO_2^-$) and p-toluenesulfinate ion (CH_3-$C_6H_4SO_2^-$), which were generated by the dissolution of NaBS and NaTS in the aqueous medium, reduced DQ to dopa by being oxidized to benzenesulfonate ion ($C_6H_5SO_3^-$) and p-toluenesulfonate ion (CH_3-$C_6H_4SO_3^-$), and that the benzenesulfonate and p-toluenesulfonate ions thus formed immediately attached to a carbon atom with the lowest electron density in the benzene ring of dopa, respectively. Therefore, these aromatic sulfinates are neither enzymatic inhibitors of tyrosinase, such as the oxalates described above, nor antioxidants, which simply reduce DQ to dopa as reported for L-ascorbic acid [7] and L-cysteine [8]. They derivatize DQ to give stable benzosulfonyl and p-toluenesulfonyl derivatives which are no longer transformed and polymerized, respectively. It has been elucidated that DQ is normally transformed to dopachrome as a direct precursor of melanin through the two independent reaction pathways by different reaction speeds [9]. The derivatization of DQ by NaBS and NaTS may occur only in the faster reaction pathway, as described above, since the inhibition of the pigmentation by these compounds were time-dependently reduced.

The derivatization method for orthoquinones employing NaBS was first developed by Norbaev, a Russian radiation biologist, who analyzed orthoquinones from polyphenolics in irradiated plant tissues [10]. In a plant radiobiochemical study, in which the author participated, 6-benzosulfonylchlorogenate dose-dependently increaseed in the NaBS-involving extracts of the irradiated *Xanthium occidentale* leaves containing chlorogenic acid [11]. In such studies, the derivatization method employing NaBS was used in the detection and determineation of orthoquinones formed by the radiochemical reactions between the polyphenol components of plants and irradiation-induced reactive oxygen species. On the other hand, various plant phenolics, such as phenolcarboxylates and flavonoids are good substrates of plant tyrosinase generally called polyphenol oxidase (PPO). The activity of PPO is significantly enhanced in the wounded plant tissues to cause browning [12], which is very inconvenient for the purification of plant proteins. The occurrence of this phenomenon is one of the major reasons why transgenic plants have not yet been widely employed in the production of useful proteins [13]. A remarkable improvement in the purity and yield of the soluble protein fraction by the reduction of browning was recently achieved by the addition of NaBS [14, 15], NaTS and/or oxalate [16] to the extraction buffer. This may be possibly due to the derivatization of ortho-quinones formed by PPO from the 3,4-ortho-diphenolcarboxylates, such as chlorogenic, caffeic and protocathechuic acids, and dopa into their stable derivatives [14-16] and also due to the inhibition of the PPO activity by oxalates [16]. It has been found that NaBS and NaTS do not react with orthoquinones from flavonoids possibly due to a streochemical disturbance, although the oxidative polymerization of chlorogenic acid as the main cause of browning in the wounded plant tissues is potently suppressed by these agents, especially by NaBS [14-16]. Therefore, it may be expected that these compounds will be able to play an important role in the field of biotechnology.

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