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Isolation and Purification of Tyrosinase Inhibitors from the Seeds of *Thuja orientalis* L.

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백자인(*Thuja orientalis* L.)으로부터 tyrosinase 저해제의 분리 및 정제

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Abstract

Previously, the methanolic extracts of thirty Korean medicinal plant seeds were screened for tyrosinase inhibitors using a rapid and simple TLC method, which was superior to a conventional spectrophotometrical in vitro assay. As a result, the methanolic extracts of *Thuja orientalis* seeds was found to have strong tyrosinase inhibitory activity. To isolate active tyrosinase inhibitors, the seeds were defatted with n-hexane under reflux, and then extracted twice with methanol under reflux at 90℃. The methanolic extract was evaporated to a small volumn in vacuo, and then successively fractionated with ether, ethyl acetate and n-butanol. The ether extract showing significant tyrosinase inhibitory activity was solubilized with 5% $NaHCO_3$ and then acidified with 6N HCl. The ether souble acidic fraction was successively chromatographed on silica gel, Sephadex LH-20 and preparative TLC. Among four compounds isolated, two of them showed stronger tyrosinase inhibitory activity, comparable to that of L-ascorbic acid (IC_{50}=28μg/㎖). These results suggest that *Thuja orientalis* seeds may be useful as potential sources of antibrowning agents in fruits and vegetables, and anti-melanoma agents in cosmetics and phamaceuticals.

Key words : *Thuja orientalis* seeds, tyrosinase inhibitors, silica gel, Sephadex LH-20, preparative TLC

Introduction

Tyrosinase(monophenol, dihydroxy-L-phenylalanine: oxygen oxidorductase, EC 1.14.18.1) is a copper-containing enzyme which catalyzes two different reactions: the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to dopa-quinone(1,2). The enzyme was mainly responsible for melanin biosynthesis (melanogenesis) in animals, and enzymatic browning (melanosis) in plants(3,4). Therefore, the specific tyrosinase inhibitors are expected to be potential antibrowning agents for the inhibition of melanosis of fruits and vegetables as well as for the prevention of local hyperpigmentation in humans, such as lentigo, melasma and freckling.

A number of tyrosinase inhibitors have been isolated from natural products, and some of them were presently used in food and cosmetic industries(5,6). 4-Hexylresorcinol is used as a functional alternative to sulfites for the control of enzymatic browning in shrimp(7-9). Meanwhile, kojic acid, arbutin, resveratrol, flavonoids and other compounds are currently utilized for treating hyperpigmentation, such as melasma and freckling in the

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cosmetics(10-14). However, the application of natural tyrosinase inhibitors to foods and cosmetics is somewhat limited due to off-flavors, toxicity, and economic feasibility(15,16). Therefore, the development of alternative safe and efficacious novel tyrosinase inhibitors is needed.

Recently, we have developed a novel TLC method for screening of tyrosinase inhibitors from natural products (17). This method was very a simple and accurate assay to search for tyrosinase inhibitors from plant sources, as compared to conventional spectrophotometrical in vitro assay. We have been screened tyrosinase inhibitors from three hundreds of Korean crude drugs using this TLC method. As a result, we found that the methanolic extract of *Thuja orientalis* seeds had several potent tyrosinase inhibitory bands.

The seeds of *Thuja orientalis* L. have been widely used as Chinese traditional medicine for the improvement of health, and the treatment of hypertensive and hemostatic diseases in Korea(18). Several studies on the chemistry and pharmacology of the seeds have been performed so far(19,20). However, no information on the isolation and identification of tyrosinase inhibitors is avaliable.

The objective of this study was to isolate and purify tyrosinase inhibitors from *Thuja orientalis* seeds.

Materials and Methods

Materials and reagents

The seeds of *Thuja orientalis* L. produced in Korea were purchased from local oriental herbal stores in Daegu, Korea. The voucher sample was deposited in my laboratory. Mushroom tyrosinase(EC 1.14.18.1; 2,750 units/㎎), L-3,4-dihydroxyphenylalanine(L-DOPA) and L-ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used in this study were of analytical grade.

Extraction and isolation of tyrosinase inhibitors

Dried and chopped *Thuja orientalis* seeds(100 g) were defatted twice with n-hexane under reflux, and then extracted twice with MeOH(2 L) under reflux at 90℃. The methanolic extract was evaporated to a small volume under reduced pressure, and successively fractionated with ether, ethyl acetate and n-butanol. The ether fraction(0.64 g) showing potent tyrosinase inhibitory activity was solubilized with 5% $NaHCO_3$ and then acidified with 6N HCl, further extracted with ether. The ether soluble acidic fraction(0.48 g) was chromatographed on silica gel (4 × 30 cm, Damstadt, Germany) with $CHCl_3$-MeOH (5:1, v/v) as eluant to give five fractions. The third fraction(0.25 g) was further applied to a Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) column(2 × 80 ㎝), and then eluted with MeOH to obtain five fractions; Frs. 1(68 mg), 2(32 mg), 3(30 mg), 4(39 mg) and 5(14 mg). The third and fourth fractions were finally purified by preparative TLC(0.5 mm, Silica gel 60, Damstadt, Merck, Germany) using $CHCl_3$-MeOH-HOAc (50:10:1, v/v) as a mobile phase to give four compounds, respectively; Comp. 1(9 mg), Comp. 2(14 mg), Comp. 3(15 mg) and Comp. 4(8 mg). The Scheme for exfraction, iselation and purifcation of tyrosinase inhibitors from *Thuja orientalis* Seeds is Shown in Fig. 1.

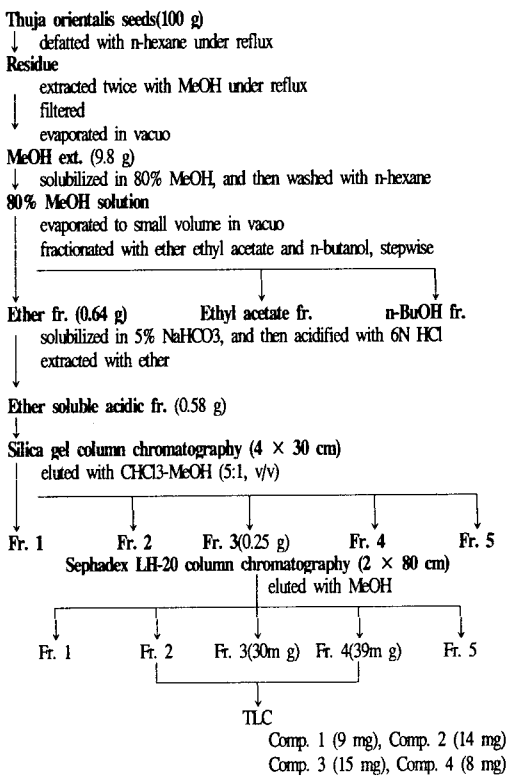

Fig. 1. Schematic procedure for extraction, isolation and purification of tyrosinase inhibitors from *Thuja orientalis* seeds.

Tyrosinase assay

Tyrosianse activity was determined by the method as described previously(21). The reaction mixture(3 mL) containing 1.52 mM L-DOPA, 67 mM phosphate buffer (pH 6.8), 90 units of mushroom tyrosinase, and the sample at various concentrations, was incubated at 25℃ for 2 min. The change in absorbance at 475 nm with or without the sample was linear with time for 2 min. The percentage inhibition of tyrosinase reaction was calculated as follows; Inhibition(%) = [(A-B)/A×100], where A represents the difference in the absorbance of the control before and after incubation, and B represents the difference in the absorbance of the test solution before and after incubation. The results were obtained from the almost concurrent three readings so that the standard deviation (S.D.) was negligible and is not shown in the results.

Results and Discussion

Tyrosinase inhibitory activity of ether, ethyl acetate and n-butanol fractions

Tyrosinase inhibitory activity of three fractions(ether, ethyl acetate and n-butanol frs.) obtained from the methanolic extracts of *Thuja orientalis* seeds is shown in Table 1. Among them, the ether fraction exhibited a potent tyrosinase inhibitory activity(IC_{50}=3 μg/mL). However, the ethyl acetate and n-butanol fractions were less activity than the ether fraction. It was reported that *Thuja orientalis* seeds contained considerable amounts of several essential oils and flavonoids(18). In particular, hinokitol and quercetin containing an α-ketol skeleton have been found to act as mushroom-tyrosinase inhibitors (22). These results suggest that essential oils and flavonoids may be mainly responsible for strong tyrosinase inhibitory effect of the ether fraction of the seeds.

Table 1. Inhibitory effects of ether, ethyl acetate and n-butanol fractions obtained from the methanolic extracts of *Thuja orientalis* seeds on mushroom tyrosinase

Compound	IC_{50}(μg/mℓ)*
Ether fr.	3
Ethyl acetate fr.	29
n-Butanol fr.	58

Average of triplicate measurements.
*IC_{50} represent the concentration causing 50% inhibition of tyrosinase activity.

Tyrosinase inhibitory activity of several fractions obtained from silica gel and Sephadex LH-20 column chromatography

Tyrosinase inhibitory activity of five fractions obtained from silica gel column chromatography is given in Table 2. Of five fractions, fraction 3 showed the most significant tyrosinase inhibitory activity(IC_{50}=8 μg/mL), followed by fraction 4, 2, 5, and 1, in descending order.

Meanwhile, five fractions obtained from Sephadex LH-20 column chromatography also showed strong tyrosinase inhibitory activity(Table 3). In particular, fraction 3(IC_{50}=12 μg/mL) and 4(IC_{50}=17 μg/mL) exhibited considerable tyrosinase inhibitory activity, but other fractions were less activity.

Table 2. Inhibitory effects of five fractions obtained from silica gel column chromatography on mushroom tyrosinase

Compound	IC_{50}(μg/mℓ)*
Fr. 1	33
Fr. 2	22
Fr. 3	8
Fr. 4	14
Fr. 5	28

Average of triplicate measurements.
*IC_{50} represent the concentration causing 50% inhibition of tyrosinase activity.

Table 3. Inhibitory effects of four fractions obtained from Sephade LH-20 column chromatography on mushroom tyrosinase

Compound	IC50(μg/mℓ)*
Fr. 1	53
Fr. 2	42
Fr. 3	12
Fr. 4	17
Fr. 5	44

Average of triplicate measurements.
*IC_{50} represent the concentration causing 50% inhibition of tyrosinase activity.

Tyrosinase inhibitory activity of four compounds obtained from preparative TLC

The tyrosinase inhibitory activity of four compounds from preparative TLC is shown in Table 4. Compound 2 (IC_{50}=26 μg/mL) and 3(IC_{50}=30 μg/mL) exhibited significant inhibitory activity, comparable to L-ascorbic acid(IC_{50}=28 μg/mL), a well-known tyrosinase inhibitor. In addition, compound 1 and 4 also showed appreciable

inhibitory activity, although their activity was weaker than that of L-ascorbic acid. Two compounds(Comp. 2 and 3) isolated have a weak absorption spectra at 230 and 265 nm(data not shown), indicating that their compounds were phenolic compounds with aliphatic branch. Some aliphatic phenolic compounds including resorcinol and salicylic acid moiety have been reported to have strong tyrosinase inhibitory activity(23,24). On the basis of published paper, these data suggest that some novel oil-like phenolic compounds, unlike flavonoids and essential oils previously known, may be mainly contribute to strong tyrosinase inhibitory effect of *Thuja orientalis* seed extracts. Further research on the structural elucidation of compound 1-4, and their inhibitory effects on enzymatic browning of several fruit juices are now in progress.

Table 4. Inhibitory effects of four compounds obtained from preparative TLC on mushroom tyrosinase

Compound	IC50(μg/mℓ)*
Compound 1	53
Compound 2	26
Compound 3	30
Compound 4	64
L-Ascorbic acid	28
Kojic acid	6

Average of triplicate measurements.
*IC_{50} represent the concentration causing 50% inhibition of tyrosinase activity.

요 약

식물추출물로부터 보다 안전하고 효과있는 새로운 tyrosinase 저해제를 개발하기 위한 연구의 일환으로 먼저 기존의 spectrophotometric assay를 개선한 새로운 TLC 검색방법을 사용하여 여러 식물추출물을 검색한 결과 백자인의 메탄올추출물이 강한 tyrosinase 저해활성을 갖고 있음을 알 수 있었다. 따라서 백자인의 메탄올추출물로부터 tyrosinase 저해제를 분리하기 위하여 먼저 탈지 메탄올추출물을 에테르, 에틸아세테이트 및 부탄올로 순차적으로 용매분획한 후 이 중 활성이 강한 에테르 추출물을 얻었다, 다음 에테르 추출물을 5% $NaHCO_3$로 씻은 후 산성화하여 얻은 강산성에테르추출물을 silica gel, Sephadex LH-20 및 분취 TLC를 각각 이용하여 4가지의 tyrosinase 저해물질을 분리하였으며, 이 중 화합물 2 (IC_{50}=26 μg/mL)와 3 (IC_{50}=30 μg/mL)은 tyrosinase 저해제로 잘 알려진 ascorbic acid (IC_{50}=28 μg/mL)와 유사하게 강한 tyrosinase 저해활성을 나타내었으며, 현재 그들의 화학구조를 동정하고 있다. 이러한 결과를 미루어볼 때 백자인으로부터 분리된 tyrosinase 저해제는 식품의 효소적갈변저해제로써 뿐만 아니라 기미, 주근깨 및 검반점을 예방할 수 있는 미백화장품 및 의약품의 신소재로써 활용될 수 있을 것으로 기대된다.

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