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Inhibition of Enzymatic Browning of Apple Juices by Benzoic Acid Isolated from Peach (*Prunus persica Batsch*) Seeds

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복숭아씨로부터 분리된 안식향산에 의한 사과주스의 효소적갈변 억제

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Abstract

Previously, the methanolic extract of peach seeds was found to have a strong tyrosinase inhibitory activity in an *in vitro* assay. Several phenolic compounds were isolated from the seeds by solvent fractionation, Sephadex LH-20 chromatography, and preparative HPLC, and one of them showing strong tyrosinase inhibition was identified as benzoic acid by UV, IR, $^{1}H/^{13}C$-NMR, and EI-MS spectroscopy. Benzoic acid (IC_{50}= 250 μg/mL) showed a considerable inhibitory effect against mushroom tyrosinase, although its activity was weaker than that of kojic acid (IC_{50}= 6 μg/mL) and L-ascorbic acid (IC_{50}=28 μg/mL), well-known tyrosinase inhibitors. In particular, benzoic acid inhibited markedly the enzymatic browning (melanosis) of apple juices at low concentration of 0.01 % and 0.05%, comparable to that of L-ascorbic acid ($P<0.05$). These results suggest that benzoic acid, one of an effective food preservatives, may be potentially useful as a functional alternative to sulfites for the control of melanosis in fruit juices

Key words : Peach (*Prunus persica Batsch*) seeds, tyrosinase and melanosis inhibitor, benzoic acid

Introduction

Enzymatic browning (melanosis) of raw fruits and vegetables is a cosmetic discoloration which has a negative impact on the appearance, consumer acceptability, commercial value, and organoleptic properties For this reason, an unfavorable enzymatic browning of many food products has been of great concern to food technologists and processors.

Tyrosinase (monophenol, dihydroxy-L-phenylalanine. oxygen reductase, EC 1.14.18.1), which is a key enzyme involved in enzymatic browning, catalyzes the hydroxylation of tyrosinase to L-DOPA and its subsequent oxidation to form dopa-quinones. Further condensation of dopa-quinones, or reaction with amino acids and proteins, leads to brown melanin pigments (1,2). Hence, a specific tyrosinase inhibitor is expected to develope as potential anti-melanosis agent.

The most widely method used in the food and beverage industries for control of enzymatic browning is the addition of sulfiting agent to foods susceptible to browning. Sulfites are currently used to inhibit the melanosis of a variety of foods including shrimp, potatoes, mushrooms, apples, and wines. However, the Food and Drug Administration has recently banned the

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use of sulfites in foods due to adverse health effects and off-flavor(3-5). Until now, a number of anti-browning treatments, including reducing agents, phenolic acids, acidulants, chelating agents, tyrosinase inhibitors, inorganic salts and enzymes, have been investigated as alternatives to sulfites but are not in commercial use except L-ascorbic acid, 4-hexylresorcinol and Sporix (6-11). Therefore, the development of new functional alternatives to sulfites is essential.

Recently, we have screened for alternative safe and efficacious tyrosinase inhibitors from plant extracts using *in vitro* assay. Among the extracts tested, peach seed was shown to have a strong tyrosinase inhibitory activity(12). In addition, peach seed oils are well-known to have anti-melanogenesis (melanin biosynthesis) activity, and widely used in cosmetics as therapeutic agents for treatment of localized hyperpigmentation in humans, such as lentigo, melasma and freckling(13,14). However, few study on the screening of major tyrosinase inhibitors from peach seeds is available.

The objective of the present study was to isolate and identify tyrosinase inhibitors from peach (*Prunus persica Batsch*) seeds, and further to investigate the effect of tyrosinase inhibitors on the melanosis of apple juices.

Materials and Methods

Materials

Peach seeds were obtained from local food factory, Hyupsung Nongsan Co. Ltd., in Taegu, Korea, and its kernels are selected, and dried before use. Mushroom tyrosinase (1.14.18.1; 4,400 units/mg), L-DOPA, kojic acid and L-ascorbic acid were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A). All other chemicals used for this study were of analytical grade.

Isolation and purification of tyrosinase inhibitor

Dried peach seeds (600 g) were extracted twice with n-hexane under reflux to remove lipid, and then extracted twice with 80% aqueous methanol under reflux. The methanolic extract was evaporated to small volumn *in vacuo*, and then partitioned successively with ether, ethyl acetate, and n-butanol. The ether soluble fraction (0.9 g) showing strong tyrosinase activity was chromatographed on a Sephadex LH-20 column (2 × 100 cm) eluting with methanol to give seven fractions: Fr. I (134 mg), Fr II (58 mg), Fr. III (368 mg), Fr. IV (134 mg), Fr. V (84 mg), Fr. VI (45 mg) and Fr. VII (27 mg). The tyrosinase inhibitory activity of each fraction was 12, 36, 83, 35, 24, 15 and 53%, respectively, at a concentration of 0.3 mg/mL. A portion (350 mg) of Fr. III was purified finally by preparative HPLC (Waters Delta Prep 4000, Boston, U.S.A) using a Nova-pak C18 column (2.5 cm × 10 cm) at a flow rate of 5 mL/min with monitored at 280 nm in a gradient solvent system: solvent A (MeOH-H_2O-TFA=20:80:0.1, v/v/v) and slovent B (MeOH-H_2O=80:20, v/v) for 55 min to give a major four compound (1, 16 mg), (2, 24 mg), (3, 15 mg) and (4, 32 mg) (Fig. 1).

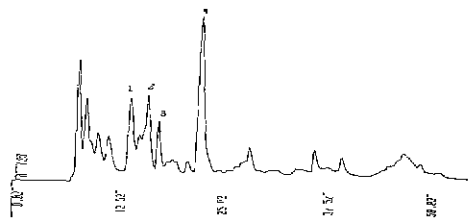

Fig. 1. HPLC chromatogram of four compounds isolated from the peach seeds.
HPLC conditions column, Novapak C_{18} (2.5 cm × 10 cm), gradient elution from solvent A (MeOH-H_2O-TFA=20:80:0.1, v/v/v) to slovent B (MeOH-H_2O=80:20, v/v) for 55 min, flow rate, 5 mL/min, detector, UV_{280} nm.

Instrumental analysis

UV-visible absorption spectrum in MeOH was recorded on a spectrophotometer (Shinco, S2030, Seoul, Korea). The IR spectrum was taken on an FS 120 HR/FRA infrared spectrophotometer (Bruker, Germany) as KBR disce, and the abosorbance frequency was expressed in cm^{-1}. ^{1}H-NMR (500MHz) and ^{13}C-NMR (125MHz) spectra were measured on a Bruker AMX-500 spectrophtometer in CD_3OD containing tetramethylsilane (TMS) as an internal standard. The electron impact-mass spectrometry (EI-MS) was determined with a Quattro II mass spectrometer (VG, U.K) at an ionization voltage of 70 eV.

Tyrosinase assay

Tyrosinase inhibitory activity was determined according

to the method of Fukushima and Kimura (14) with a slight modification. The recation mixture (3 mL) containing 1.52 mM L-DOPA, 67 mM phosphate buffer (pH 6.8), 90 units of mushroom tyrosinase, and the extracts at various concentrations, was incubated at 25℃ for 2 min. The change in absorbance at 475 nm with or without sample was calculated as follow; Inhibition(%) = [(A-B)/A×100], where A is an absorbance at 475nm after incubation with test sample. The results were obtained from the almost concurrent three reading so that the standard deviation (S.D) was negligible and is not shown in the results.

Preparation of apple juices

Apple juices were prepared from ripe Fuji apples obtained at local food stores. The apples (10 g) were cut into wedges with a kitchen appliance, and then mixed with ACE homogenizer (Nissei, Tokyo, Japan) in 0.01% and 0.05% benzoic acid solution for 3 min. The homogenate was further filtered with Whatman No. 2 filter paper under suction. Portions of juices treated by two concentrations (0.01% & 0.05%) of L-ascorbic acid were prepared like above apple juices, and used as references, while untreated portions were used as controls. The extent of browning of apple juices was calculated at intervals with colorimeter (Color JC 801, Color Techno System, Tokyo, Japan), and represented as "L" value (whiteness).

Results and Discussion

Tyrosinase inhibitory activity

The tyrosinase inhibitory effect of four major compounds isolated from peach seeds is shown in Table 1. Among four compounds tested, compound 4 (IC_{50}=250 μg/mL) showed strong tyrosinase inhubitory activity, although its activity was weaker than that of L-ascorbic acid (IC_{50}=28 μg/mL) and kojic acid (IC_{50}=6 μg/mL), well-known tyrosinase inhibitors. Meanwhile, the inhibitory effect of three other compounds was less than that of benzoic acid. These results suggest that compound 4 may be mainly responsible for a strong tyrosinase inhibitory activity of the peach seed extracts.

Table 1. Tyrosinase inhibitory effects of four compounds isolated from peach seeds

Compound	Inhibition (IC_{50}, μg/mL)
Compound 1	345
Compound 2	465
Compound 3	276
Compound 4	250
L-Ascorbic acid	28
Kojic acid	6

IC_{50} represent the concentration of compounds causing 50% reduction of tyrosinase activity.
Values are mean of triplicate measurements.
L-Ascorbic acid and benzoic acid were used as positive references.

Table 2. The detailed UV, IR, NMR and MS spectral data of benzoic acid isolated from peach seeds

Instrumental analysis	Benzoic acid
$UV_{\lambda max}$ nm (log ε)	272 (3.03)
$IR_{\gamma max}$ (cm^{-1})	2836, 1685, 1422, 1325, 1291, 931
^{1}H-NMR (acetone-d_6)	6.81 (1H, brs, -OH) 7.51 (2H, *t*, *J*=7.5 Hz, H-3 & H-5) 7.61 (1H, *t*, *J*=7.5 Hz, H-4) 8.10 (2H, *d*, *J*=7.5 Hz, H-2 & H-6)
^{13}C-NMR (acetone-d_6)	208.1 (-COOH) 131.8 (C-1) 130.5 (C-2 & C-6) 129.3 (C-3 & C-5) 127.9 (C-4)
EI-MS (*m/z*)	122, 105, 77, 51

Structural elucidation of compound 4

UV maximum absorption at 272 nm, and IR spectrum showing aromatic (2836 cm^{-1}), carboxylic (1685 cm^{-1}) and aromatic (1422-1291 cm^{-1}) groups indicated that compound 4 was a simple phenolic compound containing carboxylic group. 1H-NMR showed δ 7.51 (2H, t, J=7.5 Hz, H-3 & H-5), 7.61 (1H, t, J=7.5 Hz, H-4), 8.10 (2H, d, J=7.5 Hz, H-2 & H-6), and 6.81 (1H, brs, -OH). 13C-NMR spectrum of compound 4 was consistent with spectral data of benzoic acid (15). The EI-MS showed a molecular ion peak at m/z 122 with a base peak at m/z 77 and 105 which was deduced to benzyl and carboxylic group, respectively. Thus, compound 4 was readily identified as benzoic acid. The detailed UV, IR, ^{1}H/^{13}C-NMR, and EI-MS spectral data of compound 4 is given in Table 2. Benzoic acid was for the first time isolated and identified from peach seeds. It was found that

amygdalin, cyanogenic glycosides in the fresh peach seeds, was readily transformed into benzaldehyde by heating and emulsion (16). Thus, benzoic acid could be formed from benzaldehyde during extraction and separation procedures.

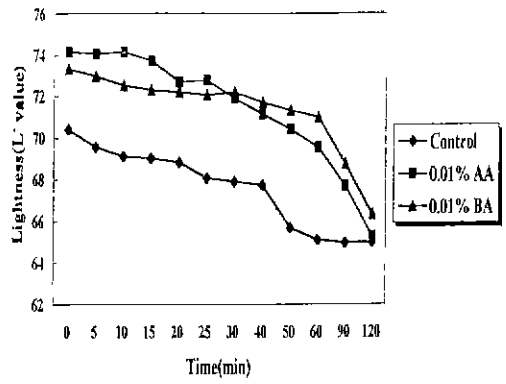

Fig. 2. Control of enzymatic browning in apple juices according to the treatments (0.01%) at 20℃. L-ascorbic acid (AA) was used as a positive reference.

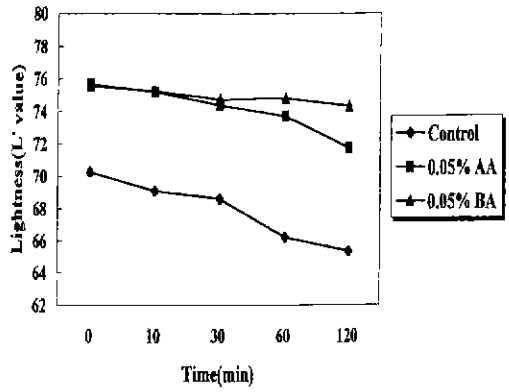

Fig. 3. Control of enzymatic browning in apple juices according to the treatments (0.05%) at 20℃. L-ascorbic acid was used as a positive reference

Inhibition of apple juices by benzoic acid

Preliminary observation of raw Fuji apple juices treated with the ether fraction of peach seeds indicated that such juices did not undergo an enzymatic browning during subsequent storage. To confirm this observation by major tyrosinase inhibitor in the ether fraction, we investigated the effect of benzoic acid, a predominant component in the ether fraction of peach seeds, on enzymatic browning of apple juices. As shown in Fig. 2 and Fig. 3, benzoic acid was very effective in preventing browning in Fuji apple juice at low concentration of 0.01% and 0.05%, comparable to that of L-ascorbic acid ($P<0.05$). In particular, with lapse of time, the inhibitory effect of benzoic acid against enzymatic browning of apple juices was superior to that of L-ascorbic acid. In addition, blanching effect for control of enzymatic browning of apple juices appeared more great in the apple juices treated with benzoic acid than in the apple juices treated with L-ascorbic acid, which is readily susceptible to oxidation during storage (data are not shown). This observation also supports earlier reports that benzoic acid, well-known as effective food preservative, acted as a potent polyphenol oxidase inhibitor (17-19), and inhibited the enzymatic browning of some fruits and vegetables (20,21). Thus, these results suggest that benzoic acid may be potentially useful as anti-melanosis agent for control of enzymatic browning of fruit juices. Further research on control of enzymatic browning of minimally processed fruits and vegetables by benzoic acid is now in progress.

요 약

여러 식물종자추출물 중 복숭아씨의 메탄올 추출물은 갈변효소, tyrosinase 저해활성이 매우 강하게 나타났다. 복숭아씨의 메탄올추출물을 용매분획, Sephadex LH-20 및 분취-액체크로마토그래피를 각각 이용하여 4가지 다른 페놀화합물을 분리하였으며, 그 중 tyrosinase 저해활성이 강한 안식향산을 UV, IR, NMR 및 MS를 이용하여 동정하였다. 안식향산의 tyrosinase 저해활성은 기존의 tyrosinase 저해제로 잘 알려진 아스콜빈산이나 코오지산 보다 낮았으나 그래도 상당한 저해활성을 나타내었다 특히 안식향산은 사과주스의 효소적갈변 현상을 크게 억제하였으며, 그 억제효과는 같은 농도에서 기존의 아스콜빈산 보다 우수하였다. 따라서 복숭아씨로부터 분리된 안식향산은 식품방부제로써 뿐만 아니라 tyrosinase 저해제로써, 여러 과실주스의 효소적갈변 현상을 억제하는 수단으로 사용할 수 있을 것으로 기대된다.

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