Eggplant Extract Inhibits Melanogenesis in B16 Melanoma Cells

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Eggplant extract markedly inhibited melanogenesis in B16 mouse melanoma 4A5 cells (B16 cells). To clarify the characteristics of the melanogenesis inhibitor in eggplant, the effective fraction, P1, was separated from the eggplant extract by ammonium sulfate fractionation and Sephadex G-100 column chromatography. The P1 fraction from the eggplant contained 63% protein, 20% neutral sugar and 11% uronic acid. P1 was stable during heating at 60°C for 30 min, and the inhibitory effect remained stable after protease digestion. P1 weakly inhibited tyrosinase activity in crude B16 cell extract in vitro, and tyrosinase activity in B16 cells cultured with P1 was reduced. The regulation of tyrosinase activity in B16 cells was equal to melanogenesis suppression in B16 cells. These results show that the eggplant extract suppresses melanogenesis in B16 cells by regulation of tyrosinase.

Keywords: B16 melanoma cells, melanogenesis, eggplant, tyrosinase

Some physiological components in foods favorably effect human physiology (Arai, 1995). Vegetables and other edible plants are rich sources of such components. Spinach, eggplant and broccoli, for example, have antimitogenicity to Salmonella typhymurium TA100 and TA98 by mutagenic compounds (Shinohara et al., 1990). Thirteen types of vegetable extracts, such as garland chrysanthemum, ginger and asparagus, indicate antioxidant activity in the measurement of betacarotene discoloration coupled with linoleic acid oxidation (Tsushida et al., 1994). The extracts of vegetables, such as the garden pea, oyster mushroom and chinese chive, promote proliferation and IgM secretion of the human-human hybridoma cell, HB4C5 cells (Kong et al., 1992). During our ongoing study on the physiological functions of vegetables, we demonstrated that nondialyzable spinach extract induced differentiation in human leukemia cells (Kobori & Shinohara, 1993; Kobori et al., 1995) and promoted the antibody secretion of human-human hybridoma cells (Baba et al., 1996). We demonstrated the effect of the eggplant extract on melanogenesis in the B16 melanoma cells of vegetable extracts (Baba et al., 1996; Shimozono et al., 1996) and apple ployphenol (Shoji et al., 1997).

Melanin pigment is synthesized in the melanocyte melanosome of the skin through oxidation of tyrosine by tyrosinase to protect the skin from ultraviolet rays (Ando et al., 1995; Friedmann et al., 1990). The resulting hyperpigmentation causes freckles, blotching and skin carcinoma. Arbutin, kojic acid (Maeda & Fukuda, 1991) and some plant compounds such as licorice extract, bearberry leaf extract (Matsuda et al., 1992) and chamomile extract (Ohmori, 1995) inhibit melanogenesis by inhibiting tyrosinase and other pathways. These compounds are used in cosmetics as skin whiteners (Akui et al., 1988; Maeda & Fukuda, 1995; Ohyama & Mishima, 1990; Kanayama et al., 1994).

In a previous study, we demonstrated that the nondialyzable extracts of eggplant, spinach, carrot and green tea markedly inhibit melanogenesis in B16 mouse melanoma 4A5 cells (Baba et al., 1996). We also showed that eggplant and radish extracts inhibited melanogenesis in B16 cells, and the inhibitory effect of the eggplant was greater than that of the radish (Iwashita et al., 1997).

In this study, we separated the active fraction from an eggplant extract that inhibited B16 cell melanogenesis and examined the heat stability and protease digestibility. We also determined the effect of the eggplant fraction on tyrosinase activity, because most skin melanogenesis depigmentation is known regulation of tyrosinase, which supports a part of the melanin synthesis pathway.

Materials and Methods

Preparation of eggplant extract Five hundred grams of eggplant (Solanum melongena L. cv. Senryo 2-gou) was purchased from a local market and homogenized in 500 ml of phosphate-buffered saline (PBS, pH 7.4) using a Warning blender. The homogenate filtered through cotton gauze was centrifuged at 10,000×g for 15 min at 4°C. The supernatant was fractionated by 0–40% (Fr.1) and 40–80% (Fr.2) saturated ammonium sulfate. Each was dialyzed against distilled water with seamless cellulose tubing and lyophilized. Fraction 2 was dissolved in distilled water containing 0.02% NaN3 and centrifuged at 10,000×g for 15 min at 4°C. The supernatant was fractionated by 0–40% (Fr.1) and 40–80% (Fr.2) saturated ammonium sulfate. Each was dialyzed against distilled water with seamless cellulose tubing and lyophilized. Protein content was determined using a DC-protein assay kit (Bio-Rad, USA), and bovine serum albumin was used as the standard protein. Neutral sugar elution patterns were measured. Protein was detected to measure the absorbance at 280 nm. Neutral sugar was detected to measure the absorbance at 490 nm by phenol-sulfuric acid reaction. Eluted fractions (P1, P2 and P3) were dialyzed and lyophilized.

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Melanogenesis assay  B16 mouse melanoma 4A5 cells (B16 cells) were obtained from the Riken Cell Bank (Tsukuba). Melanogenesis assay in B16 cells was conducted based on the method of Philip & Gilchrest (1989). B16 cells (5 x 10⁶ cells/ml) were seeded into 60 mm culture dishes and cultured in Dulbecco’s Modified Eagle Medium (D-MEM, Nissui Pharm. Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS) in a 5% CO₂ humidified incubator. After one and 3 days of seeding, the medium was replaced by fresh D-MEM supplemented with 10% FCS with samples dissolved in 100 µl PBS. After 4 days of cultivation, the cultured medium was removed and B16 cells were harvested by trypsin treatment with 1 ml of trypsin solution (200 IU/ml) and trypsin inhibitor (from soybean, Sigma, St. Louis, Mo.) solution (50 µg/ml). The harvested cell number was counted using a Micro-cell counter (CC-130, Toa Iyo Denshi Co., Ltd., Kobe). The cells were then sedimented by centrifugation, and the supernatant was removed. Cells were solubilized in 1 ml of 1 M NaOH, and the melanin content was determined by measuring absorbance at 475 nm. The amount of melanin was calculated from a calibration curve, which was determined using synthetic melanin (Sigma). Experiments were duplicated.

Heat stability of melanogenesis inhibition of eggplant extract P1  The P1 fraction was dissolved in PBS and incubated at 60°C and 100°C for 30 min in a water bath. The deposit was removed by centrifugation at 3,000 X g for 15 min. The inhibitory effect of the supernatant was compared to that of untreated P1.

Protease digestion of eggplant extract P1  P1 was dissolved in PBS and digested with protease K-agarose (Sigma) at 37°C for 72 h. During protease digestion, a small amount of the solution was collected and electrophoretically fractionated to confirm the protease digestion sufficiently. After digestion, protease K-agarose was removed by centrifuging at 3,000 X g for 15 min. The digested P1 was applied to a Sephadex G-50 column pre-equilibrated with distilled water containing 0.02% NaN₃. The inhibitory effect of the eluted fractions (F-A, F-B and F-C) on melanogenesis in B16 cells was examined.

Tyrosinase activity  B16 cells were cultured for 4 days with P1, arbutin or kojic acid. Cells were harvested and extracted in PBS containing 0.1% polyoxyethylene (10) octylphenyl ether (Triton X-100) by sonication using a Model 450 Sonifier (Branson Ultrasonics Corp., Conn., USA). Alternatively, crude tyrosinase was extracted from cells cultured without the sample, and each sample was added to it. Tyrosinase activity was assayed as dopa oxidase activity based on the method of Imokawa (Imokawa & Mishima, 1981), using 0.05% L-DOPA in PBS (pH 6.8) as the substrate. Protein content was determined using a DC-protein assay kit (Bio-Rad) and bovine serum albumin was used as the standard protein. Tyrosinase activity was revealed the percentage vs. the control.

Results  Separation of eggplant extract fraction inhibiting B16 cell melanogenesis  When 250 µg/ml medium eggplant extract (B) was added to the B16 cell medium, the melanin content in B16 cells was reduced to 64% that of control (Fig. 1). The crude extract was separated into two fractions, Fr.1 and Fr.2, by ammonium sulfate fractionation. Fr.2, fractionated using 40–80% saturated ammonium sulfate, reduced melanin content in B16 cells to 54% that of the control at a 250 µg/ml medium concentration (F), with only 19% inhibition of cell growth. Fr.1, the 0–40% saturated ammonium sulfate fraction, reduced melanin content in B16 cells to 73% that of the control at a concentration of 250 µg/ml (D), but Fr.1 apparently inhibited B16 cell growth. At 125 µg/ml of Fr.2 (E), the melanin content in B16 cells was significantly reduced without inhibiting cell growth.

Because Fr.2 inhibited melanogenesis in B16 cells without inhibiting cell growth, Fr.2 was further separated by Sephadex G-100 column chromatography (Fig. 2). After Sephadex G-100 gel filtration, Fr.2 was divided into 3 fractions: P1, P2 and P3. P1 inhibited melanogenesis in B16 cells as much as 100 µM arbutin without inhibiting cell growth (Fig. 3).
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and P3 inhibited melanogenesis, but not as much as Pl. The eggplant P1 fraction contained 63% protein, 20% neutral sugar and 11% uronic acid.

To clarify the characteristics of the melanogenesis inhibitor in eggplant, we examined the stability of P1 inhibition in heat treatment and protease digestion.

Heat stability of eggplant P1 fraction inhibiting B16 cell melanogenesis

P1 was heated at 60°C and 100°C for 30 min, and then the inhibition of heat-treated and nontreated P1 on B16 cell melanogenesis was examined. Both types of P1 reduced the melanin content of the B16 cell at a concentration of 125 μg/ml without inhibiting cell growth (Fig. 4). P1 heated at 60°C and 100°C for 30 min inhibited B16 cell melanogenesis as much as nonheated P1. Thus, the melanogenesis inhibitor in P1 was stable during heating at 60°C and 100°C for 30 min.

Protease digestion of eggplant extract P1 fraction

The stability of P1 inhibition in protease digestion was determined. P1 was digested using protease K-agarose and was separated into three fractions, F-A, F-B and F-C, using Sephadex G-50 column chromatography (Fig. 5). At a concentration of 125 μg/ml, F-A (B) inhibited melanogenesis without inhibiting cell growth (Fig. 6). This demonstrates that the melanogenesis inhibitory effect in P1 remains after protease digestion.

Effect of P1 on tyrosinase extracted from B16 cells

We examined the effect of P1 on B16 cell tyrosinase activity to clarify the mechanism of melanogenesis suppression by P1. B16 cells were cultured for 4 days under the same conditions as in the melanogenesis assay without the sample. After 4 days of cultivation, B16 cells were harvested, then crude tyrosinase was extracted from harvested cells with PBS containing 0.1% Triton X-100 by sonication. P1, kojic acid and arbutin, known as melanogenesis inhibitors, were added to the crude tyrosinase solution at a final concentration from 1 to 1,000 μg/ml. Tyrosinase (dopa oxidase) activity was measured using L-DOPA as the substrate (Fig. 7). P1 inhibited the tyrosinase activity in the B16 cell extract to 80% that of the controls at 100 μg/ml. This effect was equivalent to the effect of arbutin, whereas kojic acid inhibited 99% tyrosinase activity in the control cell extract at the same concentration.
Most of these inhibitors, such as arbutin, kojic acid, ascorbic acid, and linoleic acid, had low molecular weights (Ando et al., 1990).

The inhibitory effect of tea, sweet potato and apple on melanogenesis in B16 cells was shown in previous studies (Shimozono et al., 1996; Shoji et al., 1995). Only chlorogenic acid and other phenolic compounds inhibited B16 cell melanogenesis. In this study, we partially purified the melanogenesis inhibitor in the eggplant extract and examined its characteristics. Nondialyzable eggplant extract, which was dialyzed using seamless cellulose tubing to remove low molecular weight compounds, markedly inhibited B16 cell melanogenesis, but the eggplant ethanol extract did not (Iwashita et al., 1997). The P1 fraction inhibiting melanogenesis was separated from the nondialyzable eggplant extract, P1 contained proteins, glycoproteins and polysaccharides. These results and the chromatographic patterns on Sephadex G-100 suggest that P1 had a high molecular weight. Although P1 contained 63% protein, P1 was thermally stable, and the inhibitory activity remained after protease digestion. Because most protein was denatured by heat treatment and digested by protease, the melanogenesis inhibitor in P1 was not likely to be a protein, but more likely to be a polysaccharide with a high molecular weight.

Tyrosinase is known to be a key enzyme in melanogenesis (Ando et al., 1990). Five types of melanogenesis inhibitors in cultured B16 mouse melanoma cells have been reported and classified into two groups. One is an isolated tyrosinase-suppressive type, such as kojic acid and ascorbic acid. The other is an isolated tyrosinase-nonsuppressive type, such as placental extract and hydroquinone. The nonsuppressive type reduces tyrosinase activity in the skin. Many melanogenesis inhibitors, such as kojic acid and arbutin, suppress B16 cell melanogenesis by suppressing tyrosinase activity (Imokawa & Mishima, 1981). P1 weakly suppressed the tyrosinase activity of crude tyrosinase extract from B16 cells in vitro. The tyrosinase activity in B16 cells cultured with P1 was reduced. Tyrosinase suppression by P1 was lower than that by kojic acid but equaled that by arbutin in crude B16 cell extract in vitro. Tyrosinase reduction by P1 was lower than that by arbutin but equaled that by kojic acid in B16 cells cultured with samples. When B16 cells were cultured with 100 µg/ml of P1, melanogenesis suppression in B16 cells was also observed and nearly equaled tyrosinase reduction in B16 cells. From these results, it was demonstrated that tyrosinase regulation in B16 cells was involved in the eggplant extract depigmentation mechanism and played an important role in B16 cell melanogenesis suppression.

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**Fig. 7.** Effect of P1, kojic acid and arbutin on the activity of tyrosinase extracted from B16 melanoma 4A5 cells.

**Fig. 8.** Tyrosinase activity in B16 melanoma 4A5 cells cultured with P1, kojic acid and arbutin.

These results showed that P1 inhibited tyrosinase activity from B16 cells in vitro.

**Tyrosinase activity from B16 cells cultured with P1**

We measured tyrosinase activity in B16 cells cultured with P1, kojic acid and arbutin in the cells (Fig. 8). Adding P1 to the cultured medium of B16 cells at 300 µg/ml or less showed no influence on cell growth. Tyrosinase activity of B16 cells cultured with 100 µg/ml of P1 was 74% that of the controls, whereas the tyrosinase activity of cells cultured with 100 µg/ml arbutin or kojic acid was 27% or 81% of that of the controls. The results showed that P1 suppressed tyrosinase activity in B16 cells. The suppressive effect of P1 was lower than that of arbutin, but the same as that of kojic acid. The melanin content of B16 cells treated with 100 µg/ml of P1 was 70% of that of the controls. At 100 µg/ml, P1 inhibited melanogenesis and tyrosinase activity. These results suggested that the eggplant P1 fraction suppressed B16 cell melanogenesis by inhibiting B16 cell tyrosinase activity.

**Discussion**

To date, depigmenting agents in cultured B16 mouse melanoma cells have been found to inhibit melanogenesis. Most of these inhibitors, such as arbutin, kojic acid, ascorbic acid, and linoleic acid, had low molecular weights (Ando et al., 1990).

The inhibitory effect of tea, sweet potato and apple on melanogenesis in B16 cells was shown in previous studies (Shimozono et al., 1996; Shoji et al., 1995). Only chlorogenic acid and other phenolic compounds inhibited B16 cell melanogenesis. In this study, we partially purified the melanogenesis inhibitor in the eggplant extract and examined its characteristics. Nondialyzable eggplant extract, which was dialyzed using seamless cellulose tubing to remove low molecular weight compounds, markedly inhibited B16 cell melanogenesis, but the eggplant ethanol extract did not (Iwashita et al., 1997). The P1 fraction inhibiting melanogenesis was separated from the nondialyzable eggplant extract, P1 contained proteins, glycoproteins and polysaccharides. These results and the chromatographic patterns on Sephadex G-100 suggest that P1 had a high molecular weight. Although P1 contained 63% protein, P1 was thermally stable, and the inhibitory activity remained after protease digestion. Because most protein was denatured by heat treatment and digested by protease, the melanogenesis inhibitor in P1 was not likely to be a protein, but more likely to be a polysaccharide with a high molecular weight.

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