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# Procyanidin Trimers to Pentamers Fractionated from Apple Inhibit Melanogenesis in B16 Mouse Melanoma Cells

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The effects of apple polyphenols on melanogenesis in B16 mouse melanoma cells were investigated. The inhibitory effect of apple polyphenols was stronger than that of arbutin or kojic acid. Three polyphenol fractions (phenolic acid derivatives, procyanidins and other flavonoids) were isolated, and the procyanidins were fractionated according to the degree of polymerization using normal-phase chromatography. The procyanidin trimer-to-pentamer fractions were found to have the most pronounced effect on melanogenesis. Furthermore, each procyanidin fraction inhibited mushroom tyrosinase. No correlation between the degree of procyanidin polymerization and tyrosinase inhibitory activity was observed. Nevertheless, these observations suggest that procyanidins are effective inhibitors of tyrosinase.

**KEYWORDS: Apple procyanidins; B16 mouse melanoma cells; melanogenesis; tyrosinase**

## INTRODUCTION

Melanin is the pigment to give its characteristic color to the skin and hair along with photoprotective properties and is synthesized in the melanosomes transferred from melanocytes. Melanin biosynthesis starts with two conversions catalyzed by tyrosinase (EC 1.14.18.1), the hydroxylation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-dopa) followed by the oxidation of L-dopa to dopaquinone (*1*). In the absence of thiolic compounds, dopaquinone changes to dopachrome following reactions by 5,6-dihydroxyindol-2-carboxylic acid oxidase (TRP-2) (*2*) and dopachrome tautomerase (TRP-1) (*3*), and further polymerization reactions yield melanin pigments. Tyrosinase is the key enzyme in melanogenesis.

Melanocytes reside at the basal layer of the epidermis, and keratinocytes are layered around and above melanocytes. Ultraviolet radiation (UV) acts directly to stimulate melanin biosynthesis and the proliferation of melanocytes and also releases cytokines such as endothelin-1 (ET-1) and α-melanocyte-stimulating hormone (MSH) from keratinocytes or melanocytes (*4*). UV and cytokines or hormones induce tyrosinase level in melanocytes. In addition, reactive oxygen species are generated in the skin by UV. $H_2O_2$, which is one of the reactive oxygen species generated, causes an increase in the level of tyrosinase mRNA (*5*, *6*).

Many plant extracts, such as licorice roots (*Glycyrrhiza glabra* L.) (*7*), grape-seed extracts (*8*), and ellagic acid (*9*), have been reported to inhibit melanin biosynthesis. Furthermore, a number of natural products found in plant extracts, such as arbutin [*p*-hydroquinone-*β*-D-glucopyranoside (*10*)] and kojic acid [5-hydroxy-2-(hydroxymethyl)-4-pyrone (*11*)], are used to inhibit melanogenesis as cosmetic additives. The inhibitory mechanisms of plant extracts were reported about tyrosinase in melanogenesis. It has been reported that arbutin reduces melanogenesis by inhibiting tyrosinase biosynthesis, kojic acid and ellagic acid reduce melanogenesis through inhibiting tyrosinase itself, and *Matricaria chamomilla* extract acts as an antagonist for endothelin receptor (*6*). Natural products in plant extracts are investigated for finding new cosmetic additives.

Apple polyphenols have been reported to have a variety of biological effects, including antiallergic (*12*, *13*) and antioxidative activities (*14*). Apples contain many types of phenolic acid derivatives and flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones, and anthocyanins) (**Figure 1**) (*15*−*20*). The procyanidins consist of (+)-catechin and (−)-epicatechin units, linked together through 4→8 and 4→6 interflavonoid bonds. There are many isomeric forms depending on the extent of polymerization and the nature of the constituent units (*21*). Apple procyanidins include three dimers: procyanidin B1 [epicatechin-(4*β*→8)-catechin], procyanidin B2 [epicatechin-(4*β*→8)-epicatechin], and procyanidin B5 [epicatechin-(4*β*→6)-epicatechin], as well as the trimer procyanidin C1 [epicatechin-(4*β*→8)-epicatechin-(4*β*→8)-epicatechin] (*16*, *18*, *22*). Five additional procyanidin trimers and one tetramer have been reported recently (*20*), and Ohnishi-Kameyama et al. (*17*) have shown by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) that apple pro-

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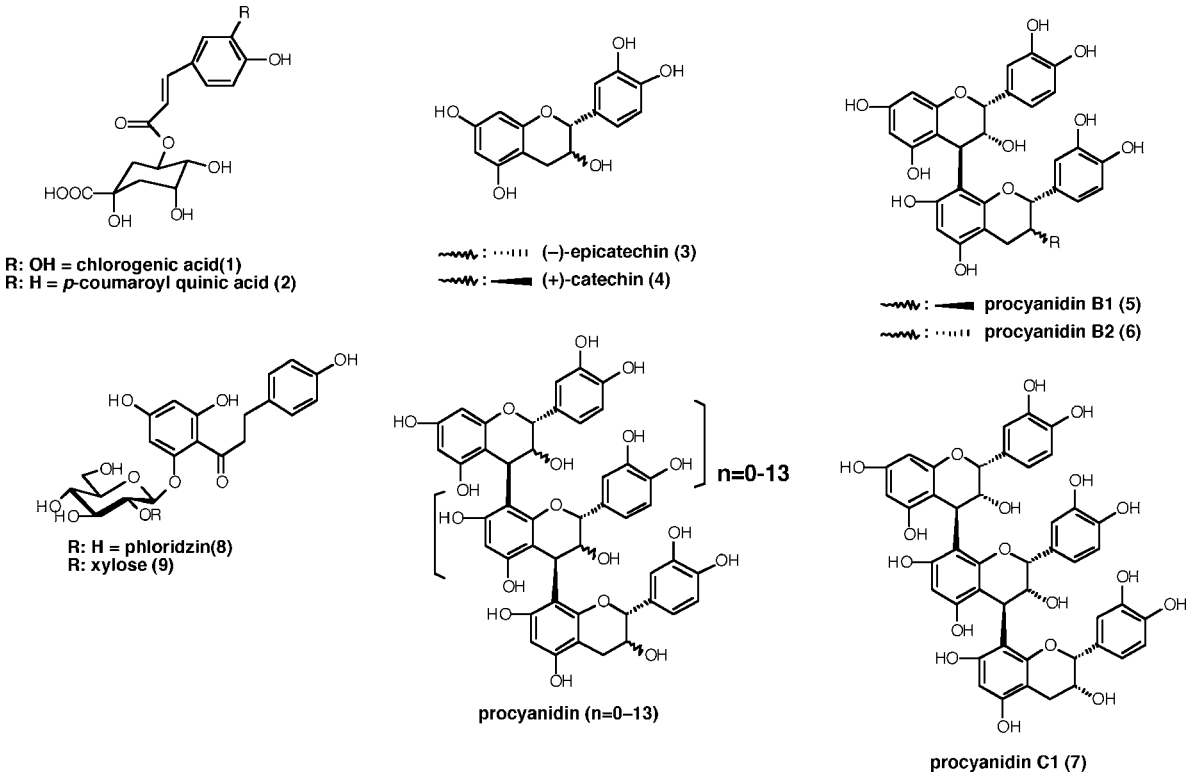


**Figure 1.** Basic structures of apple polyphenols: **1**, chlorogenic acid; **2**, *p*-coumaroyl quinic acid; **3**, (–)-epicatechin; **4**, (+)-catechin; **5**, procyanidin B1 [epicatechin-(4$\beta$→8)-catechin]; **6**, procyanidin B2 [epicatechin-(4$\beta$→8)-epicatechin]; **7**, procyanidin C1 [epicatechin-(4$\beta$→8)-epicatechin-(4$\beta$→8)-epicatechin]; **8**, phloridzin; **9**, phloretin-2′-xyloglucoside.

cyanidins are a mixture of various oligomers ranging from dimers to pentadecamers.

In this study, we examined the inhibitory effects of apple procyanidins on melanogenesis in B16 mouse melanoma cells. Furthermore, we investigated the effects of procyanidins on tyrosinase in enzymatic assay and discussed the mechanism of the inhibition of melanogenesis by procyanidins.

## MATERIALS AND METHODS

**Materials.** Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Synthetic melanin, L-glutamine, and tyrosinase were obtained from Sigma Chemical Co. (St. Louis, MO), and trypsin−EDTA, penicillin, and streptomycin were obtained from Gibco Invitrogen Co., Ltd. (Carlsbad, CA).

**Preparation of Apple Procyanidins.** Apple procyanidins were prepared according to the method of Yanagida et al. (*23*). Briefly, juice was obtained from unripe apples (*Malus pumila* cv. Fuji) and passed through a Sepabeads SP-850 column (i.d. 40 × 450 mm; Mitsubishi Kasei Co., Ltd., Tokyo, Japan). The polyphenol fraction (Fr. 1) was eluted with 50% (v/v) ethanol and concentrated by rotary evaporation at 45 °C. This fraction was a complex mixture consisting mainly of polyphenols and containing 53.6% procyanidins; the latter were made up of 8.6% dimers, 8.1% trimers, 6.3% tetramers, 4.8% pentamers, 3.5% hexamers, and 22.3% higher polymers. It also contained 9.6% flavan-3-ols (monomers), 7.0% other flavonoids, and 20.8% phenolic acid derivatives.

The polyphenol fraction (Fr. 1) was adjusted to pH 6.5 with 5 N NaOH, and 50 mL was applied to a Diaion HP-20ss column (i.d. 25 × 400 mm; Mitsubishi Kasei Co., Ltd.). The nonabsorbed fraction (Fr. 2) consisted of phenolic acid derivatives, such as chlorogenic acid and *p*-coumaroylquinic acid. After the column had been rinsed with distilled water, the procyanidin fraction (Fr. 3) was eluted with 700 mL of 25% (v/v) ethanol, and the other flavonoid fraction (Fr. 4) was eluted with 400 mL of 50% (v/v) ethanol. Fraction 3 contained flavan-3-ols [e.g., (+)-catechin and (−)-epicatechin] and procyanidins, and Fr. 4 contained phloretin glucosides (e.g., phloridzin and phloretin-2′-xyroglucoside). Finally, the eluates were concentrated by rotary evaporation at 45 °C and lyophilized (**Figure 2**).

**Fractionation of Procyanidins by Normal-Phase Chromatography.** To fractionate the procyanidins according to the degree of polymerization, we performed normal-phase chromatography using an Inertsil PREP-SIL packed column (i.d. 30 × 250 mm; GL Science, Tokyo, Japan) with hexane−methanol−ethyl acetate as the mobile phase (*24*). Procyanidin monomer-to-octamer fractions obtained were concentrated by rotary evaporation at 45 °C and lyophilized (**Figure 2**).

**Cell Lines and Cultures.** The B16 mouse melanoma cell line was obtained from the Riken Cell Bank (Tsukuba, Japan). This cell line is derived from C57BL/6J mice and is characterized by high melanin production. The cells were maintained in 10% fetal bovine serum (FBS)−DMEM, containing penicillin (100 units/mL), streptomycin (100 $\mu$g/mL), and L-glutamine (4 mM), at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air.

**Measurement of Melanin Content.** B16 mouse melanoma cells were seeded in 60 mm plates at a density of $5 \times 10^4$ cells/mL and cultured for 24 h. The medium was then replaced with fresh 10% FBS−DMEM. Each polyphenol fraction was dissolved in dimethyl sulfoxide (DMSO), and 20 $\mu$L of sample was added to cells in 5 mL of medium. After culturing for 3 days, the cells were rinsed with phosphate-buffered saline (PBS) and collected in PBS after trypsinization (0.05% trypsin in containing 0.53 mM EDTA). The harvested cells were counted with a Coulter Counter (SySmex Microcell Counter F-520; Toa Denshi Co., Ltd., Kobe, Japan) and centrifuged at 1500 rpm for 5 min. The cell pellets were taken up in 0.1% Triton X-100-PBS and sonicated. The absorbance of the extracts was measured at 475 nm, and their melanin content was calculated from a standard curve constructed with synthetic melanin. The effect of a given polyphenol fraction on melanin content per $10^6$ cells was expressed as a percentage of the content of cells cultured with DMSO alone in each experiment.

**Measurement of Tyrosinase Inhibition.** For the assay of tyrosinase, tyrosinase (Sigma Chemical Co.) obtained from mushroom was used (50000 units/mg) as used in many studies because of the convenience

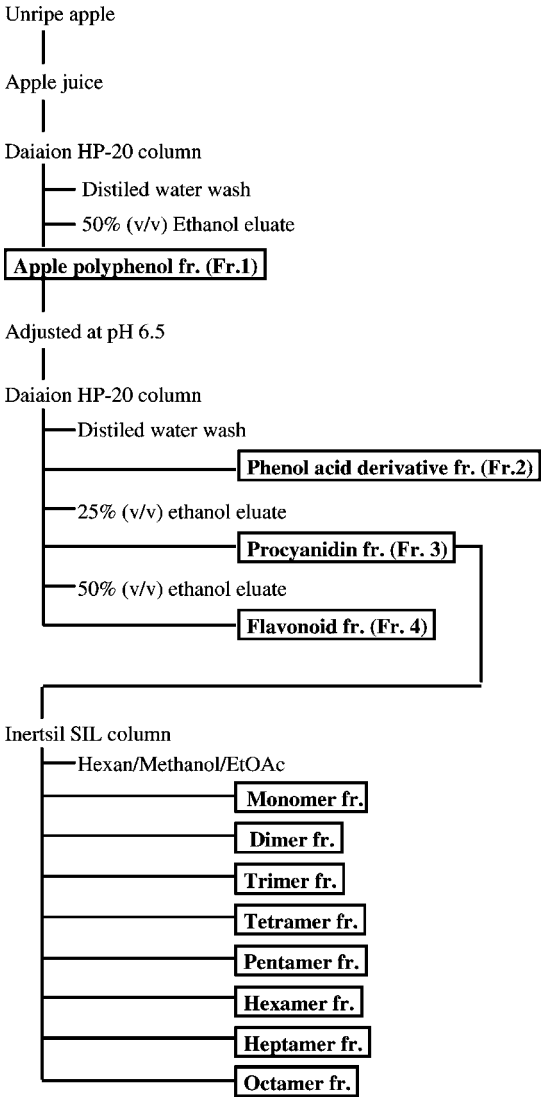


**Figure 2.** Purification of apple polyphenols and procyanidins. Apple polyphenols were prepared from unripe apples. Apple juice was passed through a Sepabeads SP-850 column (i.d. 40 × 450 mm). After the column had been washed with distilled water, apple polyphenols (Fr. 1) were eluted with 50% (v/v) ethanol. The fraction obtained was adjusted to pH 6.5 with 5 N NaOH and applied to a Diaion HP-20ss column (i.d. 25 × 400 mm), and the phenol acid derivatives (Fr. 2), procyanidins (Fr. 3), and flavonols fractions (Fr. 4) were obtained. To separate the procyanidin fraction according to the degree of polymerization, normal-phase chromatography was carried out using an Inertsil SIL packed column (i.d. 20 × 250 mm) with hexane/methanol/ethyl acetate as the mobile phase. Finally, the eluates were concentrated by rotary evaporation at 45 °C and lyophilized.

and simplicity of the assay. A sample of 0.4 mL of enzyme solution (0.75 mg/80 mL) was incubated with 0.2 mL of each procyanidin fraction in 0.1 M phosphate buffer (pH 6.8) at 37 °C for 10 min, and 0.15% L-dopa (0.4 mL) was then added. After 15 min, tyrosinase activity was determined from the absorbance at 475 nm. The percent tyrosinase inhibition was calculated as

$$\% \text{ inhibition} = (B - C)/A \times 100$$

where $A$ is the optical density (OD) at 475 nm with tyrosinase, $B$ is the OD at 475 nm with the sample and tyrosinase, and $C$ is the OD at 475 nm with the sample and tyrosinase, but without L-dopa.

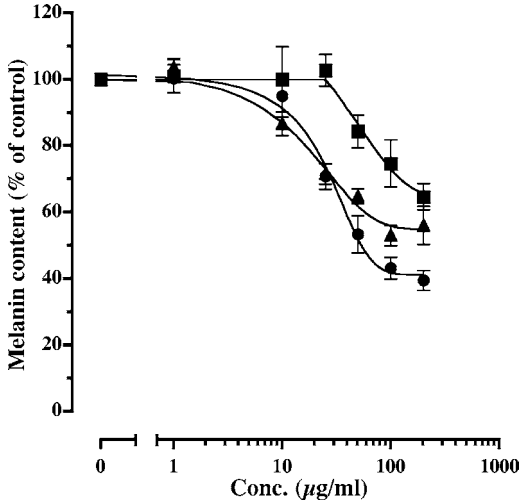


**Figure 3.** Inhibition of melanogenesis by apple polyphenols: ●, apple polyphenols; ▲, arbutin; ■, kojic acid. Apple polyphenols and cosmetic additives were added in DMSO (20 μL), and the cells were cultured for 3 days. The results are plotted as means ± SD ($n = 3$) of the percentage melanin content per $10^6$ cells relative to cells cultured with DMSO alone.

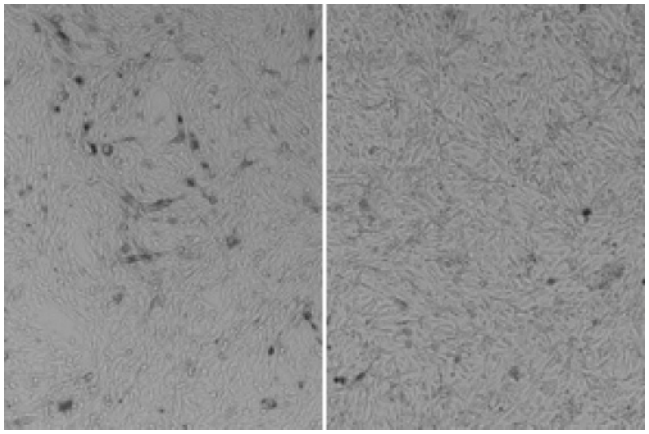

**Figure 4.** Photograph of B16 mouse melanoma cells cultured with apple polyphenols for 4 days: (left) control cells; (right) cells cultured with 100 μg/mL of apple polyphenols. Magnification ×200.

## RESULTS

**Effects of Apple Polyphenols on Melanogenesis in B16 Mouse Melanoma Cells.** As plant extracts and polyphenols inhibit melanogenesis, we investigated the effects of apple polyphenols on melanogenesis in B16 mouse melanoma cells. As shown in **Figure 3**, the apple polyphenol fraction reduced melanin production in a dose-dependent manner in the range of concentration without the inhibition of cell growth, and the melanin content was 39.5 ± 3.0% of that in the control cells at the concentration of 200 μg/mL of apple polyphenols. On the contrary, we used major cosmetic additives such as arbutin and kojic acid as positive controls. Arbutin and kojic acid reduced melanin production to 53.0 ± 3.1 and 64.7 ± 3.9% of that in the control cells at concentrations of 100 and 200 μg/mL, respectively. The apple polyphenols were more effective than arbutin or kojic acid. A photograph of cells cultured with the apple polyphenols is shown in **Figure 4**. The black spots of melanin and melanosomes in the cells can be seen to have decreased in the polyphenol-treated cells.

For the identification of active compounds in apple polyphenols, we next performed further fractionations of apple polyphenol using column chromatography. Each fraction obtained was

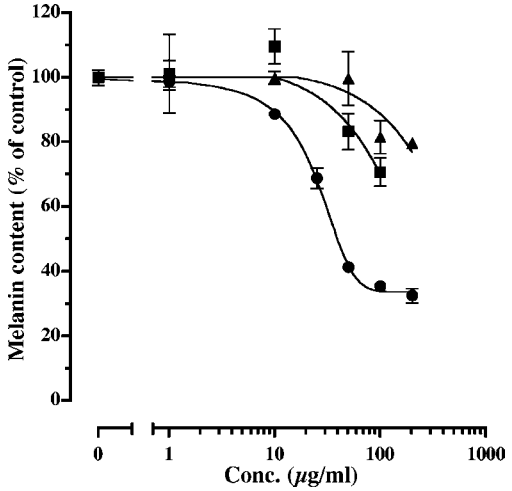


**Figure 5.** Inhibitory effect of apple polyphenol fractions on melanogenesis: ■, phenolic acid derivatives fraction; ▲, flavonols fraction; ●, procyanidins fraction. Phenolic acid derivatives, procyanidins, and flavonols fractions in DMSO (20 $\mu$L) were added, and the cells were cultured for 3 days. The results are plotted as the means ± SD ($n$ = 3) of the percentage melanin content per $10^6$ cells relative to cells cultured with DMSO (20 $\mu$L) alone.

investigated for the effect on melanogenesis in B16 mouse melanoma cells. As shown in **Figure 5**, the phenolic acid derivative fraction (Fr. 2) and the other flavonoids fraction (Fr. 4) had only a small effect on melanin production in the cells. However, the procyanidins fraction (Fr. 3) reduced the melanin content to 32.6 ± 2.2% of that in the control cells at the concentration of 200 $\mu$g/mL. These results indicated that the procyanidins, which were the major components of the apple polyphenol extract, were responsible for inhibiting melanogenesis.

**Effects of Procyanidin Oligomer Fractions on Melanogenesis.** Procyanidins in apple polyphenols are present in the mixture of oligomers and polymers ranging from dimers to pentadecamers according to the degree of polymerization (*17*, *24*, *25*). We fractionated apple procyanidins according to the degree of polymerization using normal-phase chromatography. We examined the inhibitory activity of each procyanidin oligomer fraction on melanogenesis in the cells and the cell growth (see Materials and Methods). Monomer and dimer fractions had no effect on cell growth. The trimer-to-hexamer fractions increased the cell growth to 165.6 ± 4.0, 229.3 ± 19.5, 222.2 ± 13.9, and 172.9 ± 4.5% of the control cells at the concentrations of 0.231, 0.173, 0.069, and 0.029 mM, respectively. However, the heptamer and octamer fractions strongly inhibited cell growth (**Figure 6A**).

As shown in **Figure 6B**, the monomer-to-pentamer fractions suppressed melanogenesis in a dose-dependent manner in the range of concentration without the inhibition of cell growth. The trimer-to-pentamer fractions had the strongest inhibitory effect, and the melanin contents were 34.1 ± 1.6, 36.0 ± 4.7, and 40.6 ± 4.1% of the control cells at concentrations of 0.231, 0.173, and 0.069 mM, respectively.

**Effects of Apple Procyanidins on Tyrosinase Activity.** To investigate the mechanism of inhibition of melanogenesis by the procyanidins, we examined their effect on mushroom tyrosinase. All of the oligomer fractions strongly inhibited tyrosinase activity, as did kojic acid, which is a chelator of the Cu ion in the enzyme (**Figure 7**). The $IC_{50}$ values were similar for all of the fractions: monomer, 0.074 mM; dimer, 0.235 mM; trimer, 0.140 mM; tetramer, 0.149 mM; pentamer, 0.184 mM;

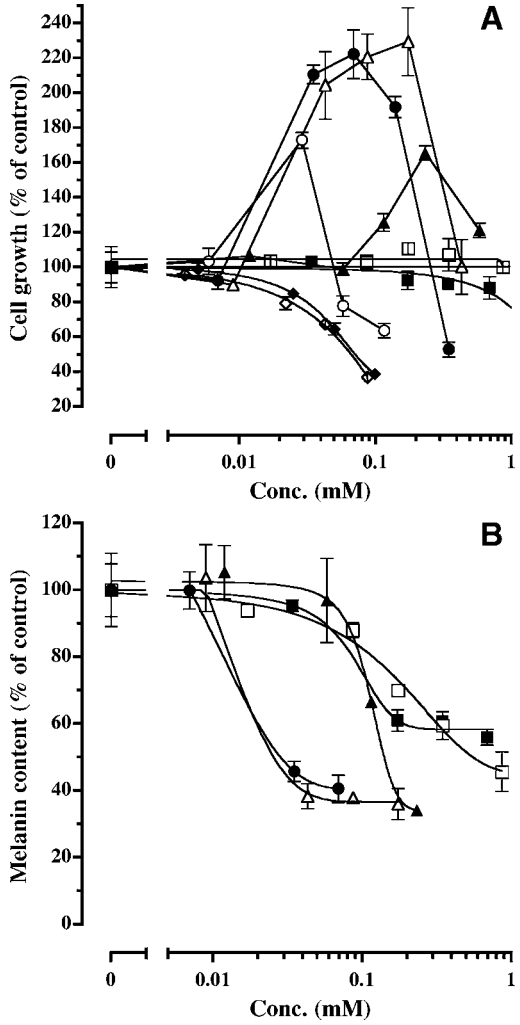


**Figure 6.** Inhibitory effects of procyanidin fractions on melanogenesis in B16 melanoma cells: (**A**) changes in B16 cell growth; (**B**) changes in melanin production in the cells; ■, monomer fraction; □, dimer fraction; ▲, trimer fraction; △, tetramer fraction; ●, pentamer fraction; ○, hexamer fraction; ◆, heptamer fraction; ◇, octamer fraction. Details are described in the legend to **Figure 5**.

hexamer, 0.127 mM; and heptamer, 0.103 mM. However, we could not observe a correlation between the degree of procyanidin polymerization and inhibitory activity on tyrosinase.

## DISCUSSION

Polyphenols are widely common secondary metabolites of plants, the content of which varies greatly between different species and cultivars and with maturity, season, region, and yield. Polyphenols are classified according to their structure as phenolic acids derivatives, flavonoids, stilbenes, or lignans (*26*). They are further subdivided on the basis of the hydroxylation of phenolic rings, glycosylation, acylation with phenolic acids, and the existence of stereoisomers.

Among the polyphenols, the proanthocyanidins, also called condensed tannins, have been reported to be present in various fruits [e.g., grape (*27*−*29*) and apple (*17*−*20*, *25*, *30*)], beverages [e.g., red wine (*28*, *31*) and beer (*32*)], and foods [e.g., grape seed (*33*−*35*), hop (*36*, *37*), barley (*38*, *39*), and cocoa (*40*, *41*)]. Proanthocyanidins are classified as procyanidins and prodelphinidin according to the flavan-3-ol units such as (+)-catechin and (−)-epicatechin or (+)-gallocatechin and (−)-epigallocatechin (*21*). The most widely studied procyanidin

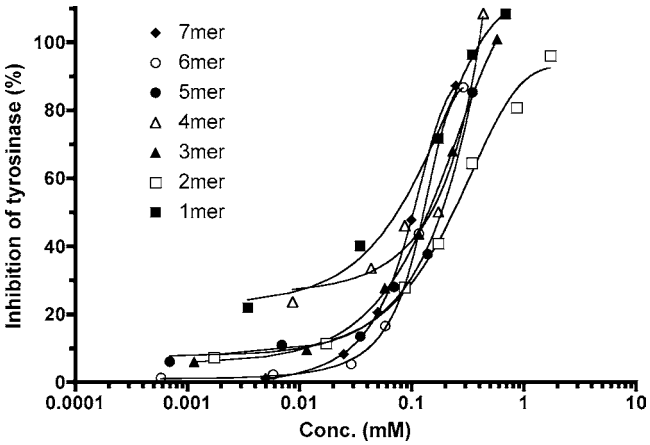


**Figure 7.** Inhibitory effects of procyanidin fractions on tyrosinase activity: ■, monomer fraction; □, dimer fraction; ▲, trimer fraction; △, tetramer fraction; ●, pentamer fraction; ○, hexamer fraction; ◆, heptamer fraction. A sample (0.4 mL) of enzyme solution (0.75 mg/80 mL) was incubated with 0.2 mL of each procyanidin fraction in 0.1 M phosphate buffer (pH 6.8) at 37 °C for 10 min, and then 0.15% L-dopa (0.4 mL) was added. After 15 min, the tyrosinase activity was determined by measuring the absorbance at 475 nm. The results are presented as the means of experiments carried out in duplicate.

B-type is linked together through the interflavanoid linkage of 4→8 or 4→6 (**Figure 1**), and the average degree of proanthocyanidin may vary widely in plants. There are many isomeric forms depending on the extent of polymerization and the nature of the constituent units (*21*).

Many plant extracts and compounds purified from plant extracts are investigated for the effects on tyrosinase and melanogenesis and used as cosmetic additives. For example, arbutin is a glycosylated hydroquinone found at high concentrations in certain plants. It has been reported that arbutin reduces melanogenesis by inhibiting tyrosinase biosynthesis (*10*). Several other natural products and polyphenols have been reported to inhibit tyrosinase (*7*, *42*, *43*) and melanogenesis (*8*, *9*, *44*, *45*).

In the present work, we found that the inhibitory effect of apple polyphenols on melanogenesis was stronger than that of kojic acid or arbutin, which are both major cosmetic additives (**Figure 3**). In addition, the procyanidins fraction of apple polyphenols had the strongest inhibitory effects on melanogenesis in the cells. Thus, procyanidins, which constitute ∼50% of the apple polyphenols, are primarily responsible for the inhibitory action on melanogenesis.

Proanthocyanidins have many functions, including antioxidative (*46*), radical scavenging (*47*), antiulcer (*48*), antidental caries (*49*), and antimelanogenic activities (*8*, *44*), the promotion of hair epithelial cell proliferation (*50*), and protection against UV rays (*51*). Many of the relevant studies have used nonpurified proanthocyanidins, because it is difficult to separate them from other polyphenols and to fractionate them according to the degree of polymerization. Hence, the identification of the active components was not clear, and the relationship between the molecular structure and degree of polymerization and the physiological activity remained unknown.

We therefore investigated which procyanidin oligomer had the strongest inhibitory activity on melanogenesis. Our results showed that the trimer-to-pentamer fractions separated by normal-phase chromatography had the strongest inhibitory effect in the following order: tetramers < pentamers < trimers. This finding defines the correlation between the degree of polymerization and inhibitory action for the first time.

Yamakoshi et al. (*8*) reported that proanthocyanidins from grape-seed extracts inhibited melanogenesis in the cells. Uehara et al. (*44*) reported that grape-seed proanthocyanidins fractionated using size-exclusion chromatography (SEC) and TLC increased the inhibitory effects on melanogenesis in B16 cells according to the degree of polymerization and that proanthocyanidins with a molecular weight exceeding that of the hexamers had the strongest inhibitory effect on melanogenesis. We have an interest in the differences in the polymerization degree of active compounds between apple procyanidins and grape-seed proanthocyanidins: apple, procyanidins trimers to pentamers; grape seeds, proanthocyanidin polymers with a molecular weight exceeding that of the hexamers. Proanthocyanidins in grape-seed extracts were reported to consist of procyanidins and polygalloyl procyanidins (*33*, *52*). It is probably suggested that the inhibitory activities are affected by differences in the cell membrane permeability and differences in interaction of proanthocyanidins with receptor and enzyme according to the degrees and structures of proanthocyanidin.

Furthermore, it is important which methods are used to separate them from other polyphenols and to fractionate them according to the degree of polymerization. Uehara et al. (*44*) used SEC by Sephadex LH-20 and TLC, but the profiles of proanthocyanidins are very complicated. The complexity is caused by proanthocyanidins with different degrees of polymerization having the same retention time in certain preparative conditions. We fractionated apple procyanidins using normal-phase chromatography clearly and characterized each fraction by LC-ESI/MS and MALDI-TOF/MS. We confirmed that apple procyanidins were fractionated according to the degree of polymerization (*24*).

To investigate the mechanism of inhibition of melanogenesis by apple procyanidins, we investigated the inhibitory effects of apple procyanidins on tyrosinase. Tyrosinase is a key enzyme in melanogenesis (*1*), along with TRP-1 (*3*) and TRP-2 (*2*). Almost all cosmetic additives, as well as tea catechins (*42*) and various flavonoids (*53*) in polyphenols, were reported to inhibit tyrosinase. Each of our procyanidin fractions proved to have an inhibitory effect on tyrosinase. However, we did not find a correlation between the degree of polymerization and the inhibitory activity on tyrosinase: the $IC_{50}$ values were similar for each of the procyanidin fractions. Therefore, it is possible that the inhibitory effect of the procyanidin trimer-to-pentamer fractions results from inhibition of the biosynthesis of tyrosinase, as well as inhibition of its activity.

ET-1 and α-MSH act directly to stimulate melanogenesis in melanocytes (*4*). α-MSH binds to the melanocortin-1 (MC-1) receptor and increases activation of tyrosinase (*54*, *55*) in melanogenesis. α-MSH increases melanin production in B16 melanoma cells. In our preliminary study, α-MSH induced melanin production in B16 melanoma 4A5 cells, but ET-1 did not affect melanin production (data not shown). It is also possible that inhibitory activities of apple procyanidins in B16 cells are affected by differences in binding activity of MC-1 receptor or α-MSH of the different procyanidin polymers.

The number of units, positions of interflavonoid bonds, and stereochemistry of the procyanidins, as well as the content of procyanidins in extracts, differ between plant species (*56*). Apple procyanidins contain the dimers procyanidin B1 and procyanidin B2 and the trimer procyanidin C1. We have shown that the main active components responsible for the antimelanogenic activity of apple procyanidins are the trimer-to-pentamer fractions. However, these fractions contain many isomers (*20*), so it will be important to separate and identify the isomers in these

fractions and to investigate the correlation between structure and inhibitory activity.

Furthermore, it is important for cosmetic additives to be safe. Apples have been eaten both raw and in processed products, such as juice, cider, brandy, jam, and vinegar, since ancient times. Although the safety of apple polyphenols has been established (*57*), further research is needed on the inhibitory mechanism of apple procyanidins.

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