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Isolation and characterization of blackish-brown BY2-melanin accumulated in cultured tobacco BY-2 cells

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ABSTRACT

The tobacco BY-2 cell line is one of the most utilized plant cell lines. After long-term culture, the cells turn brown to black, but the causal pigment is unknown. We successfully isolated a blackish-brown pigment from BY-2 cells cultured for 3 weeks. Morphological and spectroscopic analyses indicated that the pigment had similar features to a melanin-like substance reported previously. Furthermore, physicochemical analyses revealed that this pigment possessed most of the properties of melanin-like pigments. In addition, the high nitrogen content suggested that it differed from common plant melanins classified as allomelanins, suggesting a novel eumelanin-like pigment: "BY2-melanin". This is the first example showing that eumelanin-like pigments are produced in the cultures of plant cells for which the accumulation of melanin has not been reported. This tobacco BY-2 cell culture technique may represent a customizable and sustainable alternative to conventional melanin production platforms, with significant potential for industrial and pharmacological applications.

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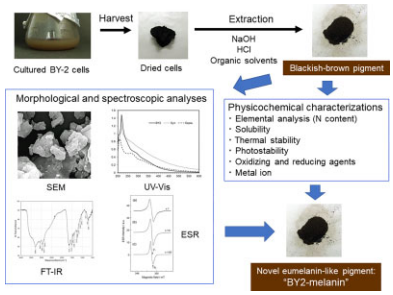
Graphical Abstract



Morphological and spectroscopic analyses and physicochemical characterizations revealed that "BY2-melanin" isolated from BY-2 cells was a novel eumelanin-like pigment.

Keywords: melanin, tobacco, BY-2, cultured cell, tyrosinase

Plant pigments include chlorophylls, flavonoids (anthocyanins, chalcones, flavones, etc.), carotenoids, and betalains (betacyanins, betaxanthins, etc.) (Grotewold 2006; Tanaka, Sasaki and Ohmiya 2008), which reflect light of a specific wavelength, and they have the function of energy conversion or protecting cells from harmful ultraviolet rays, while by giving color to leaves and flowers, they attract animals, including insects and humans (Grotewold 2006). On the other hand, brown to black pigments, including melanin-like pigments, are also present in the seed coats of common plants (Glagoleva, Shoeva and Khlestkina 2020).

Melanin is a generic name for the dark pigment accumulated in animal and human hair, skin, and eyes; it is a useful substance that absorbs ultraviolet rays to prevent DNA damage (Solano 2014). Animal melanin includes not only brown to black "eumelanin" but also orange-red "pheomelanin", and "neuromelanin" is a dark pigment that accumulates in the brain (Solano 2014; d'Ischia *et al.* 2015). Eumelanin is synthesized by the polymerization of dopachrome transformed from dopaquinone, which is obtained through tyrosine and/or L-dihydroxyphenylalanine (L-DOPA) oxidation by tyrosinase (Figure S1, left). Pheomelanin is an orange-red pigment found in red hair and feathers of birds, originally formed similarly to eumelanin, but L-DOPA undergoes the integration of cysteine in the polymer (cysteinylation), and therefore, it contains sulfur (Figure S1, center). On the other hand, neuromelanin is a mixture of eumelanin and pheomelanin. In addition to its protective effect against ultraviolet rays, melanin has unique properties such as the ability to capture radicals and resistance to temperature, so it is expected to be used for electronics such as electrodes and photovoltaic cells (Paulin and Graeff 2021). Furthermore, it has recently been shown to have physiological activity such as antitumor (El-Obeid *et al.* 2006), anti-inflammation (Mimura *et al.* 1987; Oberg *et al.* 2009), and antiallergy (Kawamoto *et al.* 2019), indicating that melanin has the potential to be used as a lead compound for new drugs (d'Ischia *et al.* 2015).

In addition to animals, melanin is also found in fungi and eubacteria (Singh *et al.* 2021). Eumelanin and pheomelanin are found in fungi and bacteria (Figure S2, center), while allomelanin, a nitrogen-free heterogeneous category of melanin-like polymers, is often found in fungi (Figure S2, left). They are derived from many sources, including catechols, 1,8-dihydroxynaphthalene (DHN), and so on (Figure S2, left). Several fungi and eubacteria have been shown to produce a special allomelanin, pyomelanin, which is derived from homogentisic acid (HGA) (Figure S2, right). Pyomelanin found on the surface of eubacteria seems to be involved in protection from oxidative stress and electron transfer (Solano 2014; d'Ischia *et al.* 2015). Melanin-like pigments are also found in plants. Black inert organic substances, commonly found in the crust-like covering of some seeds of Asparagales and Asteraceae called "phytomelanin", seem to provide resistance to insect feeding and drought (Pandey and Dhakal 2001). However, it is also present in a broader range of common plants, and many have been reported to accumulate allomelanin as plant melanin (Glagoleva, Shoeva and Khlestkina 2020), such as watermelon (Nicolaus *et al.* 1964), tea (Sava *et al.* 2001), tomato (Downie *et al.* 2003), fragrant olive (Wang *et al.* 2006), chestnut (Yao, Qi and Wang 2012), morning glory (Park 2012), sesame (Panzella *et al.* 2012), black oat (Varga *et al.* 2016), black garlic (Wang and Rhim 2019), barley (Shoeva *et al.* 2020), and Nigella (Hassib 1998). Plant allomelanin is also called catechol melanin because the most common precursor in plants is catechol (Solano 2014). Despite many reports, the synthetic pathway of plant melanin is still poorly studied. So far as known, it is mainly associated with the enzymatic reaction that is involved in the chloroplast-located polyphenol oxidases (PPOs). PPOs belong to a family of Cu-containing oxidoreductase that can act on phenols in the presence of oxygen, and they seem not only to accept monophenols (cresolase) and/or *o*-diphenols (catecholase) as substrates but also to be involved in oxidative polymerization (Mayer 2006, Figure S1, right). These enzymes released from the chloroplast interact with vacuolar phenolic substrates, forming highly reactive *o*-quinones, which polymerize into melanin (Glagoleva, Shoeva and Khlestkina 2020, Figure S1, right).

In cultured plant cells, oxides of phenolic substances have been reported as causes of browning and blackening (Bhat and Chandel 1991; Jones and Saxena 2013), but melanin accumulation is also presumed (Kaur *et al.* 2018). For example, some tissue culture textbooks state that the blackening of media and cultures during the culture of plant cells is due to the production of melanin (Onay and Jeffree 2000; Kaur *et al.* 2018). However, although it has been shown that melanin could be produced in cultured cells of Nigella, which accumulate plant melanin (Haseeb and Elhag 2012), there are no reports, including in the textbooks mentioned above, that show melanin as the brown to black pigment found in cultured cells and media.

The tobacco (*Nicotiana tabacum*) cultured cell line BY-2 (referred to as "BY-2 cells") is widely known to plant cytologists

and molecular biologists and is the most commonly used model cell line in the world (Nagata, Nemoto and Hasezawa 1992; Nagata, Sakamoto and Shimizu 2004). This cell line was originated from seedlings of *N. tabacum* cv. Bright Yellow 2 in 1968 by Dr. N. Kawashima of the Japan Tobacco and Salt Public Corporation (now Japan Tobacco, Inc.) for industrial use (Kato *et al.* 1972). Due to its high proliferation ability, it was initially used for research to produce raw materials for cigarettes (Kato *et al.* 1976), and, later, cloned cells derived from the BY-2 cell line showed a high accumulation of ubiquinone 10 (coenzyme Q_{10}), much more than whole tobacco leaves (10-fold, Ikeda, Matsumoto and Noghchi 1976). On the other hand, BY-2 cells are one of the most suitable materials for basic research on plant cells, being composed of a single cell or a cluster of several cells. This excellent trait is ideal for observing the cell cycle in synchronized culture, analyzing stage-specific gene expression, and examining the cytoskeleton (Kumagai-Sano *et al.* 2006). Furthermore, BY-2 cells can be used for transient expression and transformed cells because it is easy to isolate protoplasts and introduce foreign genes. In addition, since the cell mass is small and homogeneous, it is also used for observing the subcellular localization of proteins using green fluorescent protein (GFP) (Kumagai-Sano *et al.* 2006). Previously, BY-2 cells were mainly donated by Prof. T. Nagata of the University of Tokyo, but, at present, it is deposited at the Experiment Plant Division of the RIKEN BioResource Research Center (BRC) in Japan for distribution (https://epd.brc.riken.jp/en/pcellc).

BY-2 cells turn brown (rarely black) after long-term incubation in solid or liquid cultures, as seen in tissue cultures of other plants, but there are no reports of melanin accumulated in these cells and media. Therefore, to investigate whether melanin-like pigments accumulate in BY-2 cells, we isolated a blackish-brown pigment from BY-2 cells and analyzed its morphology and spectroscopic and physicochemical properties.

Materials and methods

Cells, media, chemicals, and reagents

Tobacco BY-2 cells (Nagata, Nemoto and Hasezawa 1992; Nagata, Sakamoto and Shimizu 2004) were kindly given by Prof. Y. Machida, Nagoya University, in 1998 and have been maintained by weekly subcultures in our laboratory. The BY-2 cells were used as an extraction material for BY2-melanin. Linsmaier–Skoog (LS) liquid medium (Linsmaier and Skoog 1965) supplemented with 0.2 mg/L 2,4-D (LSD) was used to culture BY-2 cells.

Synthetic melanin (M8631, Sigma-Aldrich, St. Louis, MO, USA) and Sepia melanin (derived from *Sepia officinalis*, M2649, Sigma-Aldrich) were used for comparison. Unless otherwise noted, chemicals and reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) or Fujifilm Wako Pure Chemical Corp. (Osaka, Japan).

Culture of BY-2 cells

Every 7 days, 3 mL of BY-2 cells were subcultured in a 300 mL flask containing 75 mL of LSD liquid medium and cultured at 130 rpm on a gyrotory shaker (Model G-10, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 25 °C in the dark. To supply the extract source of BY2-melanin, 1 mL of BY-2 cells cultured for 1 week were transferred to a 100 mL flask containing 20 mL of LSD liquid medium and cultured under the above conditions for 3 weeks.

Extraction of BY2-melanin

The extraction procedure for BY2-melanin was constructed by modifying the method commonly used for melanin extraction (Pralea *et al.* 2019). BY2-melanin was extracted from 3-week-old cultured BY-2 cells. First, BY-2 cells were dried continuously for 48 h at 75 °C. Five grams of the dried BY-2 cells were suspended in 120 mL of 0.5 M NaOH, and incubated at 75 °C for 3 h with stirring every 1 h. After incubation, the suspension was centrifuged at 10 000 $\times g$ for 10 min at 22 °C. After centrifugation, the blackish-colored supernatant was collected, and the remaining pellet was washed with 120 mL of 0.5 M NaOH and centrifuged. This procedure was repeated (~4 times) until the BY-2 cells completely lost their blackish color, resulting in the BY2-melanin completely dissolved in 0.5 M NaOH. The blackish supernatants containing BY2-melanin were combined (final volume ~480 mL) and passed through a double filter paper to completely remove BY-2 cell debris.

The blackish solution containing BY2-melanin was acidified with 6.0 M of HCl, adjusted to pH 2.0, and incubated for 2 h at room temperature with stirring. The solution was centrifuged at 10 000 $\times g$ for 10 min at 22 °C, and the blackish pellet was collected.

To hydrolyze the carbohydrates and proteins, the blackish pellet was thoroughly dispersed in ~40 mL of 6.0 M HCl using a vortex mixer and incubated for 2 h at 100 °C (Varga *et al.* 2016; Li, Chen and Tang 2018). After treatment, the suspension was centrifuged at 10 000 $\times g$ for 10 min at 22 °C. The pellet was thoroughly dispersed in 40 mL of distilled water (DW), then centrifuged again at 10 000 $\times g$ for 10 min at 22 °C, and the pellet was collected. To remove fatty acids and other substances, the collected pellet was dispersed in 30 mL of 100% EtOH using a vortex mixer, agitated for 30 min on a shaker at room temperature, and centrifuged at 10 000 $\times g$ for 10 min at 22 °C. After centrifugation, the pellet was collected and then thoroughly dispersed and washed in 30 mL of DW using a vortex mixer. After centrifugation at 10 000 $\times g$ for 10 min at 22 °C, the pellet was collected. Following the same procedure as described above, the collected pellet was treated with ethyl acetate and acetone, then washed with DW by centrifugation at 10 000 $\times g$ for 10 min at 22 °C.

Finally, the recovered pellet was collected in a 2 mL screw-cap tube as BY2-melanin and lyophilized in a freeze dryer (FD-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to obtain a blackish-brown fine powder. After its dry weight was measured, BY2-melanin was stored in the shade at −20 °C.

Optical microscopy and scanning electron microscopy

A small amount of dried BY2-melanin and Sepia melanin (Sigma-Aldrich) in a 1.5 mL microtube was thoroughly ground with a pellet mixer (homogenizer) and suspended in a small amount of ethanol. A drop of the suspension was placed on a slide glass, the ethanol evaporated, and the dry matter was observed with an optical microscope (Optiphot-2, Nikon Co., Tokyo, Japan). Observed images were taken with a CCD camera (DS camera system, Nikon).

For scanning electron microscopy (SEM) observation, the same ethanol-suspended BY2-melanin was left to stand for 30 min, and then one drop of the supernatant was placed on a cover slip (about 6–7 mm square) and air-dried. The samples were placed on a sample holder and coated with platinum using a JFC-1600 coater (Jeol, Tokyo, Japan), after which they were subjected to SEM (JSM-5610LV, JEOL).

Ultraviolet-visible light absorption spectrum

The extracted BY2-melanin was dissolved in 0.5 M NaOH at 0.05 mg/mL, and the absorbance was scanned with an ultraviolet-visible (UV-Vis) spectrophotometer (DU730, Beckman Coulter, Fullerton, CA, USA) at a wavelength of 200–600 nm. For comparison, synthetic melanin (Sigma-Aldrich) and Sepia melanin (Sigma-Aldrich) were used at the concentration of 0.05 and 0.025 mg/mL, respectively.

Elemental analysis

The BY2-melanin was analyzed for its composition of elements using a CHNS/O analyzer (2400 II, PerkinElmer, Inc., Shelton, CT, USA).

Fourier-transform infrared spectroscopy

Fourier-transform infrared (FT-IR) spectroscopy (Spectrum One, PerkinElmer, Inc.) was performed using a diffuse reflection cell to investigate IR active stretching modes. For the measurements, all the samples were diluted to 2 mass% by potassium bromide (KBr).

Electron spin resonance spectroscopy

The powder samples (5 mg) were placed in electron spin resonance (ESR) Suprasil quartz tubes (OD 5 mm). ESR spectra were measured on an Elexsys E500 (X-Band, Bruker Co., Billerica, MA, USA) CW spectrometer operating at a 100 kHz modulation frequency and a microwave power of 1 mW. A typical spectroscopic frequency used for the measurements was about 9.787 GHz. Line-shape simulations of ESR spectra were generated to precisely evaluate g-values and linewidths using WinEPR SimFonia software (ver. 1.25, Bruker).

Solubility test of BY2-menalin

HCl, NaOH, KOH, and NH_4OH solutions were used as acids and alkalis at concentrations of 0.05–1.0 M. Hexane, toluene, ethyl acetate, chloroform, dichloromethane, acetone, pyridine, ethanol, dimethyl sulfoxide (DMSO), and methanol were used as organic solvents with different polarities. In addition, polyethylene glycol monoethyl ether (Cellosolve) (Lea 1952) and Soluene-350 (PerkinElmer, Inc., Waltham, MA, USA; Wakamatsu and Ito 2002) were used as organic solvents for which melanin dissolution has been reported. The following were used as buffers: 0.5 M sodium acetate (pH 5.2), 0.5 M sodium citrate (pH 7.0), 0.5 M MOPS-KOH (pH 7.0), 0.5 M HEPES-KOH (pH 7.5), 0.5 M Tris–acetate (pH 7.9), and 0.5 M Tris–HCl (pH 8.0) (Kawamoto *et al.* 2019).

The solubility test was slightly modified with reference to the method using filter paper by Kawamoto *et al.* (2019). One milligram of BY2-melanin in 100 µL of various solvents (final concentration 10 mg/mL) was suspended with a vortex mixer and left at room temperature for 24 h. Ten-millimeter square grids were made on filter paper (Whatman 3MM Chr, Cytiva, Marlborough, MA, USA), the BY2-melanin suspension was stirred with a vortex mixer, and 1 µL of each supernatant was spotted in the center of the grid. Next, 1 µL of the supernatant centrifuged at 10000 $\times g$ for 15 min was also spotted. For quantification of solubility, 1 µL of each supernatant was spotted on three grids of another filter paper. After drying, these filter papers were scanned with an optical scanner (V700 photo scanner, Epson, Nagano, Japan), and spot images were captured in 48-bit color or 16-bit grayscale using Epson scanning software. These spot images were analyzed by ImageJ (National Institute of Health: https://imagej.nih.gov/ij/). To quantify the solubility, the black and white of the grayscale image were inverted, and the density (brightness) was quantified as pixels.

Thermal stability test of BY2-melanin

Three milliliters of BY2-melanin dissolved in 0.5 M NaOH at a concentration of 0.05 mg/mL was placed in a 13.5 mL glass vial with a lid (Mighty vial No. 4, Maruemu Co., Osaka, Japan) and incubated at 25, 50, 75, and 100 °C. The absorbance (λ_{218}) was measured hourly for 0–5 h.

Photostability test of BY2-melanin

For the fading by light, 1 µL of 10 mg/mL BY2-melanin dissolved in 0.5 M NaOH was spotted on a filter paper (Whatman 3MM Chr) with grids, the same as in the solubility test. For comparison, 1 µL of 1 mg/mL synthetic melanin (Sigma-Aldrich) dissolved in 0.5 MNaOH was also spotted. These filter papers were placed under a solar lamp (R-62 Sunlamp 60 W, Kyokko Electric Industrial Co., Ltd., Tokyo, Japan), a UV lamp (GL15, Toshiba Lighting & Technology Inc., Kanagawa, Japan), or in the dark at 25 °C, and after 0, 1, 3, 5, and 10 days, they were scanned with an optical scanner (V700 Photo scanner, Epson) to capture images of the spots. Color fading was evaluated by digitizing the density of the captured spots with ImageJ to compare the spot density at each measurement with that at the start of irradiation [density ratio (DR)].

In addition, a glass Petri dish with a diameter of 30 mm containing 2 mL of BY2-melanin dissolved in 0.5 M Tris–HCl (pH 8.0) at a concentration of 0.05 mg/mL was sealed with UV transmissive film (Titer Stick HC film, Fukae-Kasei Co., Ltd., Kobe, Japan) to prevent evaporation, placed under a solar lamp, a UV lamp, or in the dark at 25 °C. The distance from the light source was 30 cm. Absorbance of λ_{max} (212 nm) was measured daily for 0–5 days.

Reaction to oxidizing and reducing agents

The effect of the oxidizing agents was examined with $KMnO_4$, $K_2Cr_2O_7$, NaOCl, and H_2O_2 using 0.5 mg/mL BY2-melanin solution in 0.5 M Tris–HCl (pH 8.0), 0.1 M HCl, or 0.5 M NaOH. $KMnO_4$ (0–1 mM), $K_2Cr_2O_7$ (0–2 mM), NaOCl (0%–0.5%), or H_2O_2 (0%–7.5%) were added to the melanin solution and left in the dark at room temperature for 2–24 h to be measured by absorbance and visual observation. BY2-melanin derivatives produced by these oxidizing agents showed strong absorption in a wide range of UV wavelengths, including the absorption peak of the original BY2-melanin, resulting in interference with the quantitative measurement of BY2-melanin. The range of wavelengths unaffected by the derivatives was 350–500 nm (Kannan and Ganjewala 2009), while in part of this range, the colors of $KMnO_4$ (brown) and $K_2Cr_2O_7$ (yellow) prevented their quantification. Therefore, 450 nm was selected as the best wavelength for the quantitative measurement of BY2-melanin.

For the reducing agent effect, Na_2SO_3 (0–50 mM) was added to BY2-melanin (0.5 mg/mL) in 0.5 M NaOH and left in the dark at room temperature for 2 h, after which the absorbance (λ_{218}) was measured and the visual fading was examined.

To confirm the reaction for polyphenols (Li, Chen and Tang 2018), 0–1.0 mg/mL BY2-melanin solutions in 0.1 M Tris–HCl

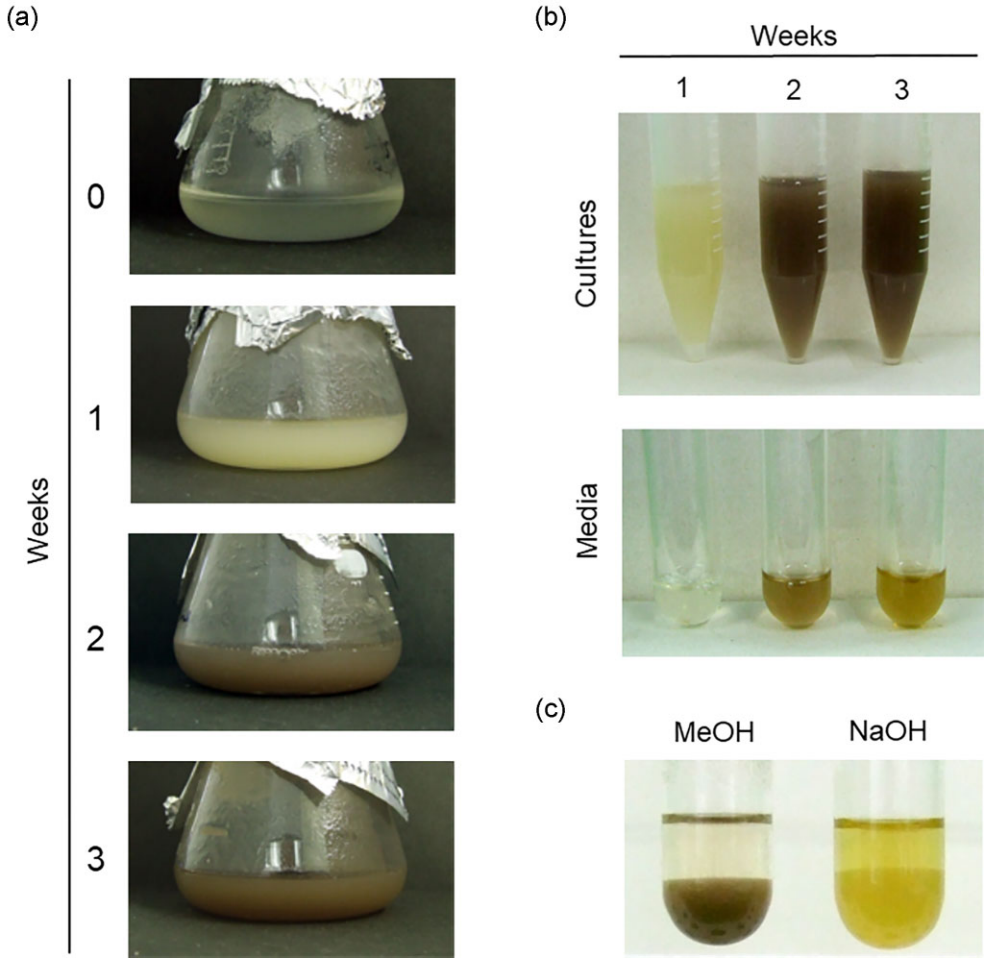


Figure 1. Accumulation of blackish-brown pigment in BY-2 cells. (a) The appearance of brown to black discoloration in the flasks of cultured BY-2 cells. The numbers on the left indicate the culture period (weeks). (b) Close-up view of BY-2 cells. Top, cultures; bottom, media. The numbers on the upper side indicate the culture period (weeks). (c) Extraction of a blackish-brown substance from BY-2 cells cultured for 3 weeks. Left, MeOH; right, 0.5 M NaOH. In the test tube, the upper layer is the solvent, and the lower layer is the cell debris. The blackish-brown pigment in the cells was extracted into the solvent only with 0.5 M NaOH, but it appeared yellow due to its low concentration.

(pH 8.0) were added with $FeCl_3$ to 50 mM and left at room temperature for 24 h to display precipitation.

Reaction to metal ions

The reaction to metal ions was carried out with $CuSO_4$, $MgSO_4$, $CaCl_2$, Na_2SO_4, $FeCl_3$, $AlCl_3$, and $ZnCl_2$. Each chemical was added to a 0.05 mg/mL BY2-melanin solution in 0.1 M NaOH to 0 or 0.1 mM, and the absorbance (λ_{218}) was measured after 0 and 24 h.

Tyrosinase inhibitor test

Tyrosinase inhibition tests were performed using two well-known but different types of inhibitors, kojic acid (KA) and phenylthiourea (PTU) (Zolghadri *et al*. 2019). KA and PTU were administered to the BY-2 cells in 20 mL LSD medium immediately or 7 days after transfer to final concentrations of 0–2.5 mM and 0–1.0 mM, respectively, and then the BY-2 cells were cultured in the same way as preparing the melanin extraction materials. The effect of the inhibitors was confirmed for up to 2 weeks after transfer.

Statistical analysis

The experimental results were the mean and standard deviation of three samples, and each experiment was repeated at least twice. Statistical significance was determined by paired *t*-test and assigned at $P < .05$, but high statistical significance was assigned at $P < .01$.

Results

Accumulation of blackish-brown pigment in BY-2 cells

In a typical BY-2 cell subculture in our work, 3 mL of cultured cells in 75 mL of LSD medium in a 300 mL flask with shaking were repeatedly transferred to 75 mL of fresh LSD medium in a 300 mL flask every week. Such cultured cells did not turn brown in 2 weeks but were mostly brown 3 weeks after transfer (data not shown). On the other hand, when 1 mL of cells cultured for a week was transferred to 20 mL of LSD medium in a 100 mL flask and cultured with shaking, the cells began to turn brown at around 10 days and completely turned blackish-brown 3 weeks after transfer (Figure 1a and b). The medium also turned blackish-brown (Figure 1b), suggesting that some type

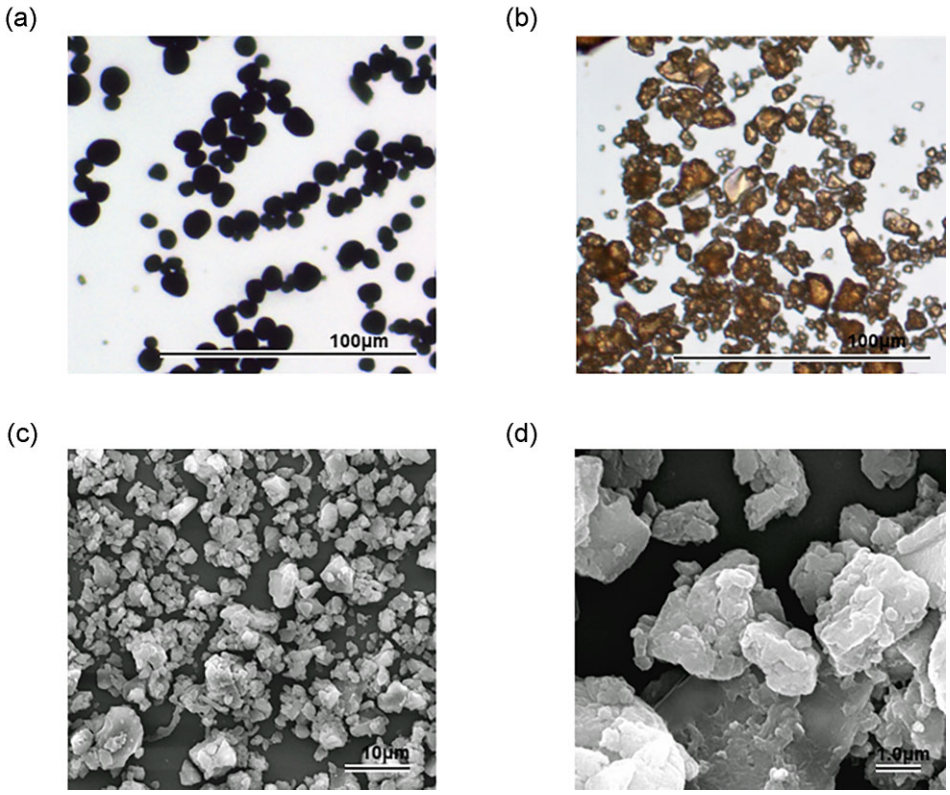


Figure 2. Morphology of BY2-melanin. (a) Optical microscope image of Sepia melanin for comparison. (b) Optical microscope image of BY2-melanin. (c) SEM image of BY2-melanin. (d) Higher magnification SEM image of BY2-melanin. Black bars indicate 100 μm (a and b), 10 μm (c), and 1.0 μm (d), respectively.

of blackish-brown pigment was produced or accumulated inside the cells and secreted or leaked out from the cells. To identify this blackish-brown pigment, the 3-week-old cultured cells were harvested, crushed, and extracted with organic solvents such as ethanol and methanol, but no blackish-brown pigment was eluted from these solvents (Figure 1c, left). However, the blackish-brown pigment was dissolved in 0.5 M NaOH, indicating that it could be extracted from the cells with this solution (Figure 1c, right). The sites and conditions of accumulation of blackish-brown pigment in the BY-2 cells were analyzed separately and will be reported elsewhere.

Isolation of blackish-brown pigment

As described in Materials and Methods, the blackish-brown pigment was eluted from the harvested and dried cells (Figure S3a) with NaOH and insolubilized with HCl. Subsequent degradation of residual carbohydrates and proteins was performed in HCl at 100 °C, followed by treatment with organic solvents to remove lipids and other substances. The isolated pigment formed soft lumps after lyophilization but turned into a fine powder when crushed well (Figure S3b). More than ten independent extractions routinely yielded 150–170 mg of blackish-brown pigment from 5 g of dried BY-2 cells. Hereafter, the isolated blackish-brown pigment is termed "BY2-melanin".

Morphology of BY2-melanin

The morphology of lyophilized and ground BY2-melanin was observed by optical microscopy and SEM. In the optical microscopic observation, Sepia melanin (Sigma-Aldrich) was used for comparison. Under the optical microscope, Sepia melanin was observed as black 3–10 μm spheres, which matched well with the morphology and size of quasi-spherical clusters observed under low-magnification SEM (Figure 2a; Mbonyiryivuze *et al.* 2015). BY2-melanin was not in the form of black spherical particles like Sepia melanin but was instead dark brown amorphous particles of an irregular size (Figure 2b). SEM observations revealed that the BY2-melanin particles were angular and irregular in size, with large particles approximately 10 μm and small particles approximately 2 μm (Figure 2c and d). Such amorphous and similar-sized melanin particles have also been reported in wide-ranging organisms such as dung beetles (*Catharsius molossus* L.; Xin, Tan and Yang 2015), fungi (*Apiosporina morbosa*; Singla, Htut and Zhu 2021), and mushrooms (*Pleurotus* spp.; Zhang, Wu and Huang 2022).

UV-Vis spectrum analysis

The BY2-melanin dissolved in 0.5 M NaOH was scanned at wavelengths from 200 to 600 nm to measure the absorbance spectrum. Synthetic melanin (Sigma-Aldrich) and Sepia melanin (Sigma-Aldrich) dissolved in 0.5 M NaOH were used as references. The spectrum of synthetic melanin showed a gradual increase in absorbance from 600 nm, a linear increase from 400 to 230 nm, and a sharp increase from 230 nm, followed by a peak at 218 nm (Figure 3, Syn). The spectrum of Sepia melanin showed that the absorbance gradually increased from around 500 nm, linearly increased from 400 to 280 nm, then increased slightly sharply; a low peak was seen around 265 nm, and after a sharp increase from around 230 nm, two peaks were observed at 210 and 206 nm (Figure 3, Sepia). For BY2-melanin,

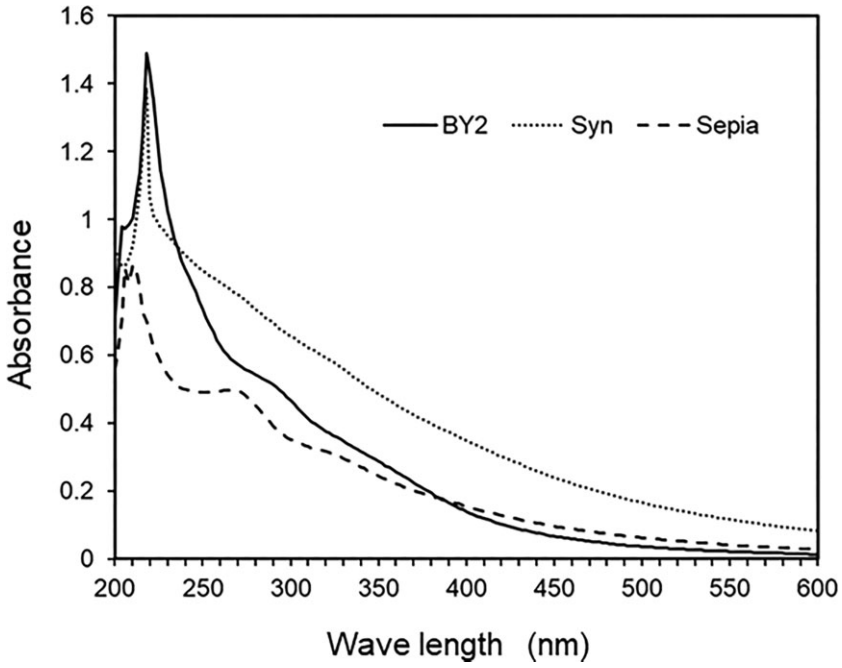


Figure 3. UV-Vis spectra of three melanins. BY2-melanin (0.05 mg/mL), synthetic melanin (0.05 mg/mL), and Sepia melanin (0.025 mg/mL) dissolved in 0.5 M NaOH, respectively. UV-Vis spectra of BY2-melanin (BY2, solid line), synthetic melanin (Syn, dotted line), and Sepia melanin (Sepia, dashed line) were measured by scanning from 600 to 200 nm with a spectrophotometer.

Table 1. Elemental analysis of BY2-melanin in two independent extractions

	Elements (%)				
Extraction	C	H	N	S	C/N molar ratio
Extract 1	54.31	4.24	10.65	0	5.95
Extract 2	54.30	4.24	10.63	0	5.96

the absorbance gradually increased from around 500 nm, followed by a linear increase from 400 to 300 nm, and then a sharp increase. Subsequently, a shoulder was observed around 280 nm, and after a rapid increase from around 265 nm, a peak was observed at 218 nm (Figure 3, BY2). These results clarified that BY2-melanin has an absorbance spectrum similar to that of the synthetic and Sepia melanins reported so far (Pralea *et al.* 2019), although the peak wavelength is slightly different.

Elemental analysis

BY2-melanins obtained by two independent extractions had almost the same ratio of constituent elements and contained C, H, and N but not S (Table 1), suggesting that BY2-melanin was different from pheomelanin, which contains sulfur. The N content of BY2-melanin was about 10%, suggesting that this melanin was different from allomelanin reported as plant melanin (Solano 2014). Also, when evaluated only from the C/N molar ratio (~6.0), the BY2-melanin was similar to mushroom (*Pleurotus* spp.) melanin (5.6–6.1; Zhang, Wu and Huang 2022), although it contained a small amount of sulfur.

FT-IR analysis

The spectrum of BY2-melanin (Figure 4) obtained by FT-IR was analyzed with reference to the interpretation by Coates (2000) and the FT-IR absorption band list described in Pralea *et al.*'s review (2019). The FT-IR spectra of BY2-melanin were also compared with those of synthetic and Sepia melanins. The FT-IR spectrum of BY2-melanin was similar to those of synthetic and Sepia melanins, with broad absorption bands at 3700–2500 and 1800–900 cm^{-1}, and slightly broader absorption bands at 800–500 cm^{-1}, but many small absorption peaks were also found, suggesting a more complex structure than those of synthetic and Sepia melanins (Figure 4; Figures S4a and S4b). A detailed analysis revealed that the broad absorption band at 3600–2800 cm^{-1} was due to stretching vibrations (O–H and N–H) of the amino, amide, or carboxylic acid, phenolic, and aromatic amino functions present in the indolic and pyrrolic systems (Wang and Rhim 2019). In particular, a relatively strong absorption at 3366–3167 cm^{-1} centered at 3286 cm^{-1} indicated O–H and N–H stretching vibrations (Sava *et al.* 2001; Dong and Yao 2012; Zhang, Wu and Huang 2022). A weak broad absorption at 3125–3046 cm^{-1} centered at 3065 cm^{-1} was an aromatic C–H stretching, supported by the presence of aromatic ring bands at 1692–1607 and 1563–1503 cm^{-1} (Coates 2000). Two relatively strong peaks at 2961 and 2937 cm^{-1} and a small peak at 2874 cm^{-1} were considered to be the stretching vibration of aliphatic C–H groups (Beltran-Garcia *et al.* 2014; Singla, Htut and Zhu 2021). In the next broad absorption band at 1800–900 cm^{-1}, three strong peaks, including 1631 cm^{-1}, were considered to be aromatic C=C stretching and COO-symmetric stretching (Yao, Qi and Wang 2012), which were also seen in synthetic melanin (Figure S4a). The strong peaks around 1515 cm^{-1} were considered to be N–H deformation and C–N stretching (amide II band) and aromatic C=C stretching (Yao, Qi and Wang 2012), and similar peaks were observed in Sepia melanin (Figure S4b). The smaller peak at 1456 cm^{-1} was attributed to aliphatic C–H deformation (Yao, Qi and

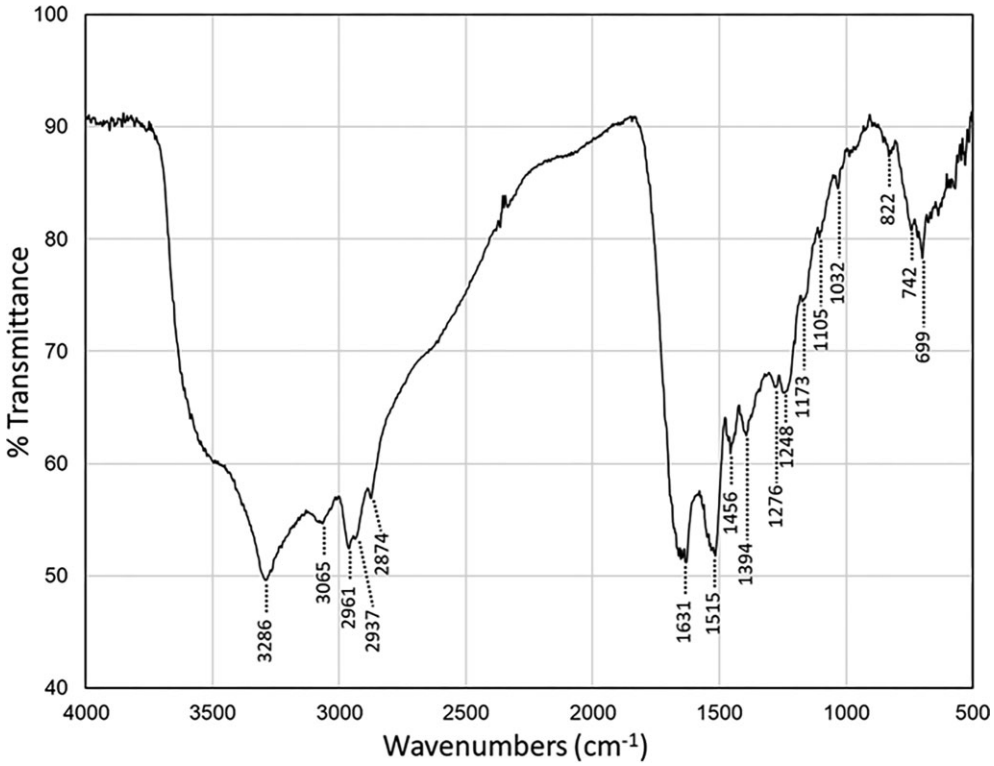


Figure 4. FT-IR spectrum of BY2-melanin. Numerical values indicate the wavenumbers (cm^{-1}).

Wang 2012), and the next smaller peak at 1394 cm^{-1} was attributed to phenolic C–O–H bending and indolic and phenolic N–H stretching (Xin, Tan and Yang 2015; Song *et al.* 2016). Two smaller peaks at 1276 and 1248 cm^{-1} were considered to be stretching vibrations of carboxyl groups (Hu *et al.* 2015). There were very small peaks at 1173, 1105, and 1032 cm^{-1} in the absorption slope at 1225–900 cm^{-1}, which were considered to be asymmetric stretching vibration of C–O–C (Ye *et al.* 2014; Liu *et al.* 2018; Ye *et al.* 2019), also seen in synthetic melanin and Sepia melanin (Figures S4a and S4b). In the smaller absorption band of 800–500 cm^{-1}, a small but sharp peak was observed at 699 cm^{-1}, which was considered to be aromatic C–H out-of-plane bending, along with 822 and 742 cm^{-1} (Coates 2000). Small peaks in the lower absorption band were likely due to alkene C–H substitution (El-Naggar and El-Ewasy 2017; Wang and Rhim 2019) and were also observed in both synthetic and Sepia melanins (Figure S4a and S4b).

ESR analysis

ESR spectra measured for the powder samples in air at room temperature are shown in Figure 5. The parameters of the spectra are presented in Table 2. The ESR spectrum of BY2-melanin consisted of an isotopic singlet with no hyperfine structure at $g = 2.0038$ and a peak-to-peak linewidth (ΔH_{pp}) of 0.70 mT (Figure 5a). On the other hand, the synthetic melanin showed an isotropic singlet line at $g = 2.0029$ with a ΔH_{pp} of 0.56 mT, and the Sepia melanin showed a singlet having a small anisotropy in the g-value, $g_{\perp} = 2.0043$ and $g_{\parallel} = 2.0019$, with a ΔH_{pp} of 0.32 mT (Figure 5b and c). The ESR parameters of BY2-melanin are very close to those of the synthetic and Sepia melanins and are in agreement with those previously reported for melanin radicals (Mason, Ingram and Allen 1960; El-Obeid *et al.* 2006; Yao, Qi and Wang 2012; Varga *et al.* 2016). Based on the double integration of the first-derivative ESR spectra, the relative ratio of the radical concentration per unit weight for the three samples was estimated as follows: BY2-melanin : synthetic melanin : Sepia melanin = 1 : 5.5 : 16. The radical concentration in BY2-melanin was the lowest of the three.

Solubility analysis

Two different analytical methods were used to determine the solubility of BY2-melanin in various solvents more quantitatively.

In the first method, the solubility was confirmed by the presence of insoluble matter. The BY2-melanin was added to various solvents at a concentration of 10 mg/mL and dissolved for 24 h. The supernatant obtained by centrifuging these solutions and the solution resuspended by vortex mixing were spotted on the same filter paper. The lower the solubility in the solvent tested, the greater the number of insoluble clumps found in the suspension spots on the filter paper. The solubility results for 10 mg of BY2-melanin in 1 mL of each of the alkaline solutions, acid solutions, organic solvents, and buffers are shown in Figure S5.

From the above results, since some amount of BY2-melanin was dissolved depending on the solvent and its concentration, the solubility was quantified by the color intensity of the BY2-melanin solutions in various solvents. That is, the solution was colored in correlation with the amount of dissolved BY2-melanin, reflected in the intensity of the color when spotted on the filter paper. Therefore, in the second method, 0–10 mg/mL of BY2-melanin solutions in 0.5 M NaOH with complete solubility were spotted on filter paper (Figure S6a). Next, to quantify the color intensity as the spot density, the images captured with an optical scanner were quantified as the pixel density of

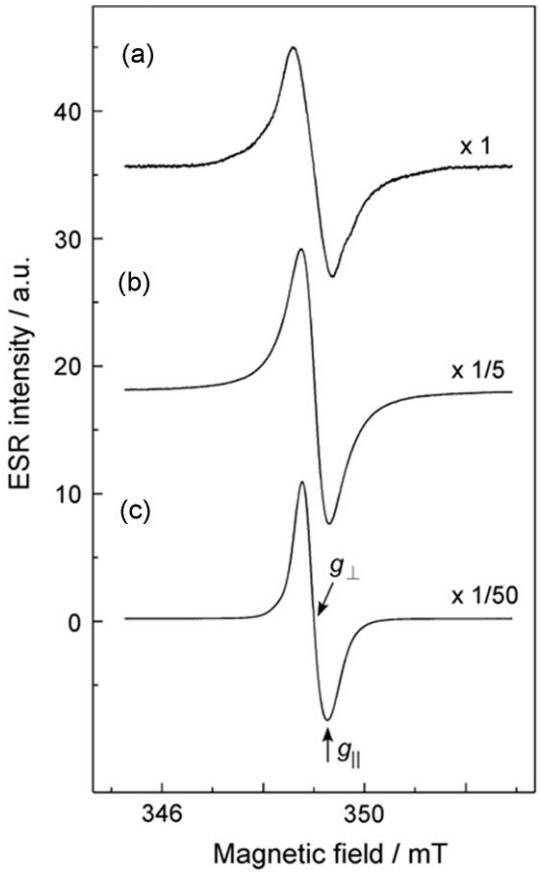


Figure 5. ESR spectra observed for powder samples at room temperature. (a) BY2-melanin; (b) synthetic melanin; (c) Sepia melanin. The approximate positions of $g_\parallel$ and $g_\perp$ are indicated with arrows.

Table 2. g_{iso}-values and ΔH_{pp}[a] of EPR signals observed for powder samples of BY2-melanin and reference melanins at room temperature

Sample	g_{iso}	ΔH_{pp}/mT
BY2	2.0038	0.70
Synthetic	2.0029	0.56
Sepia	2.0035	0.32
	$g_\perp = 2.0043$[b]	
	$g_\parallel = 2.0019$[b]	

[a]Peak-to-peak linewidth of the first-derivative ESR spectrum.
[b]$g_{iso} = (2 \times g_\perp + g_\parallel)/3$.

each spot using ImageJ. This method revealed a positive linear correlation between the concentration of the BY2-melanin and the density of the spots (Figure S6b). To quantify the solubility of BY2-melanin in various solvents, samples were prepared in the same manner as in the first method, and 1 µL of each supernatant after centrifugation was spotted on filter paper, and the spot images were captured for analysis by ImageJ.

In alkaline and acid solutions, 10 mg of BY2-melanin was well dissolved in 1 mL of 0.05–1.0 M NaOH, KOH, and NH_4OH. (Figure S5a, Table 3). Despite its strong acidity, the BY2-melanin was modestly dissolved with HCl at the concentrations of 0.05 (3.6 mg/mL), 0.1 (4.9 mg/mL), and 0.3 M (0.9 mg/mL), but was hardly dissolved at 0.5 M or more (Figure S5b, Table 3).

The solubility of BY2-melanin was investigated in ten organic solvents with different polarities and in water. Except for a small amount (3.5 mg/mL) of dissolution in DMSO, the BY2-melanin was insoluble in all the other organic solvents tested, regardless of the polarity. Also, it was hardly soluble in water (Figure S5c, Table 3). In addition, the BY2-melanin was not dissolved in Cellosolve (ethylene glycol monoethyl ether), which was reported to dissolve hair melanin (Lea 1952). Although it was well soluble in Soluene-350 (Wakamatsu and Ito 2002), the solution was slightly darker in color and strongly viscous and thus could not be used as a solvent for various experiments (Figure S5c, Table 3).

The solubility of BY2-melanin in six different compositions and pH buffers was investigated. The BY2-melanin was hardly dissolved in a weakly acidic sodium acetate buffer (pH 5.2) and a neutral sodium citrate buffer (pH 7.0). On the other hand, the BY2-melanin was moderately soluble in neutral MOPS-KOH buffer (pH 7.0, 7.0 mg/mL) and weakly alkaline Tris–acetate buffer (pH 7.9, 8.1 mg/mL), while it completely dissolved in weakly alkaline HEPES-KOH buffer (pH 7.5) and Tris–HCl buffer (pH 8.0), as well as 0.5 M NaOH (Figure S5d, Table 3). From these results, depending on the solute, the BY2-melanin was soluble in neutral to weakly alkaline buffers. In particular, the high solubility of BY2-melanin in HEPES-KOH buffer suggests that, like squid ink melanin, it can be administered to animal cells to observe various physiological effects (Kawamoto *et al.* 2019).

Thermal stability

To examine the thermal stability, the BY2-melanin was incubated at four different temperatures of 25, 50, 75, and 100 °C for 5 h. There was almost no decrease in absorbance after 5 h at any temperature, indicating stability even at a high temperature of 100 °C for at least 5 h (Figure 6).

Photostability

BY2-melanin (10 mg/mL) and synthetic melanin (1 mg/mL) were spotted on the filter paper and irradiated with a solar light lamp (60 W) or a UV lamp (15 W, 253 nm), or left in the dark as a control. In 10 days, both the BY2-melanin and the synthetic melanin showed no color fading in the dark and slight color fading under the solar light lamp without a statistically significant difference ($P < .01$) compared to the initial color intensity (Figures 7a and S7a). On the other hand, fading was observed in both melanins under UV irradiation. The BY2-melanin rapidly faded to 0.65 (DR) after 1 day, 0.4 (DR) after 3 days, then gradually faded, and after 10 days, faded from 0.2 (DR) until a faint brown color was observed (Figure 7a). Although the fading was slightly slower, the synthetic melanin faded to 0.2 (DR) after 10 days, similar to the BY2-melanin (Figure S7b).

In addition, when the denaturation of BY2-melanin in solution was confirmed by the absorbance (λ_{212}), almost no denaturation was observed in the dark and under the solar light lamp, the same as in the filter paper test (Figure 7b). However, with UV irradiation, the absorbance decreased immediately after 1 day, continued to decrease, and was completely denatured after 5 days (Figure 7b).

Reaction to oxidizing and reducing agents

The effects of $KMnO_4$, $K_2Cr_2O_7$, NaOCl, and H_2O_2 on the oxidation reaction of BY2-melanin were investigated by visual fading and a decrease in absorbance. Two hours after adding 0–1.0 mM of $KMnO_4$, visual evaluation was obscured by the color of $KMnO_4$ (data not shown), but the absorbance clearly

Table 3. Solubility of BY2-melanin in various solvents

Solvent	Chemical	Concentration (M)	Solubility (mg/mL)	Remarks
Alkaline solution	NaOH	0.05–1.0	≥10	
	KOH	0.05–1.0	≥10	
	NH_4OH	0.05–1.0	≥10	
Acidic solution	HCl	0.05	3.61 ± 0.74	
		0.1	4.89 ± 0.31	
		0.3	0.90 ± 0.34	
		≥0.5	Insoluble	
Organic solvent	Hexane		Insoluble	Polarity: 7.3
	Toluene		Insoluble	Polarity: 8.9
	Ethyl acetate		Insoluble	Polarity: 9.1
	Chloroform		Insoluble	Polarity: 9.3
	Dichloromethane		Insoluble	Polarity: 9.7
	Acetone		Insoluble	Polarity: 9.9
	Pyridine		Insoluble	Polarity: 10.2
	Ethanol		Insoluble	Polarity: 11.2
	DMSO		3.51 ± 2.72	Polarity: 12.0
	Methanol		Insoluble	Polarity: 12.9
	Cellosolve		Insoluble	
	Soluene-350		≥10	
	H_2O		Insoluble	Polarity: 21.0
Buffer solution	Sodium acetate	0.5	Insoluble	pH 5.2
	Sodium citrate	0.5	Insoluble	pH 7.0
	MOPS-KOH	0.5	6.96 ± 0.27	pH 7.0
	HEPES-KOH	0.5	≥10	pH 7.5
	Tris–acetate	0.5	8.13 ± 2.51	pH 7.9
	Tris–HCl	0.5	≥10	pH 8.0

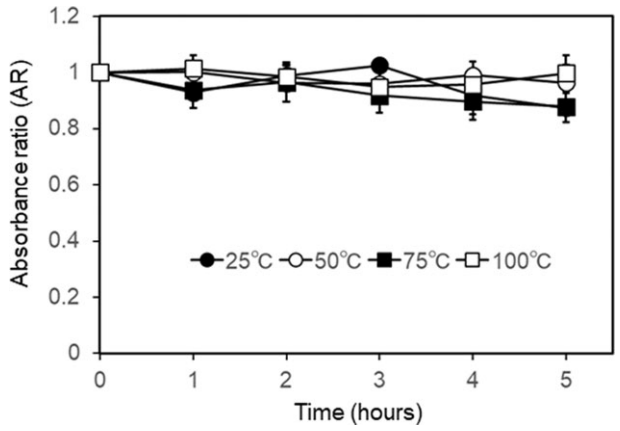


Figure 6. Temperature stability of BY2-melanin. BY2-melanin dissolved in 0.5 M NaOH at a concentration of 0.05 mg/mL was incubated at 25 °C (black circles), 50 °C (white circles), 75 °C (black squares), or 100 °C (white squares) for 5 h, and the absorbance (λ_{218}) was measured every hour. The ratio of the absorbance at the start to that at each hour [absorbance ratio (AR)] was determined. Data points represent the mean (markers) ± standard deviation (bars) acquired in triplicate. All data are representative of three independent experiments.

decreased in a concentration-dependent manner (Figure 8a). Similarly, in the case of $K_2Cr_2O_7$, the visual fading was not clear for the yellow color (data not shown), but the absorbance clearly decreased in a concentration-dependent manner (Figure 8b). With the addition of NaOCl, clear color fading was visually observed in a concentration-dependent manner after 2 h, and almost complete fading was observed at 0.5%, which was also supported by the decrease in absorbance (Figures 8c and S8a). The addition of H_2O_2 also decreased the color of BY2-melanin in a concentration-dependent manner, and the absorbance decreased as well (Figures 8d and S8b). These results indicated that the BY2-melanin was oxidized and faded by an oxidizing agent.

In the reduction reaction, Na_2SO_3 was added to a 0.5 mg/mL BY2-melanin solution to a concentration of 0–50 mM, and after 2 h, visual observation and absorbance (λ_{218}) measurements were performed, but there was no change (Figures 8e and S8c). This result indicated that the BY2-melanin does not react to reducing agents.

Furthermore, 0–1.0 mg/mL of BY2-melanin was added to 50 mM of $FeCl_3$, and it was confirmed whether brown precipitation, a positive reaction for polyphenols, occurred after 24 h (Li, Chen and Tang 2018). A fine brown flocculent precipitate was observed in the solution of BY2-melanin (data not shown). After centrifugation at 10 000 ×*g* for 10 min, it became clear that the amount of collected precipitate increased in correlation with the concentration of BY2-melanin (Figure S8d). This feature, one of the chemical properties of melanin, also supported that the BY2-melanin was a melanin-like substance (Selvakumar *et al.* 2008; Zhan *et al.* 2011; Li, Chen and Tang 2018).

Reaction to metal ions

The BY2-melanin was reacted with a solution containing 0.1 mM of $CuSO_4$, $MgSO_4$, $CaCl_2$, Na_2SO_4, $FeCl_3$, $AlCl_3$, or $ZnCl_2$ for 24 h, and the changes in absorbance were examined. As shown in Figure 9, the absorbance did not change for Cu^{2+}, Mg^{2+}, Ca^{2+}, Na^+, Al^{3+}, and Zn^{2+} but decreased by 10% for Fe^{3+} with statistical significance ($P < .01$), suggesting the possibility of changes such as decomposition.

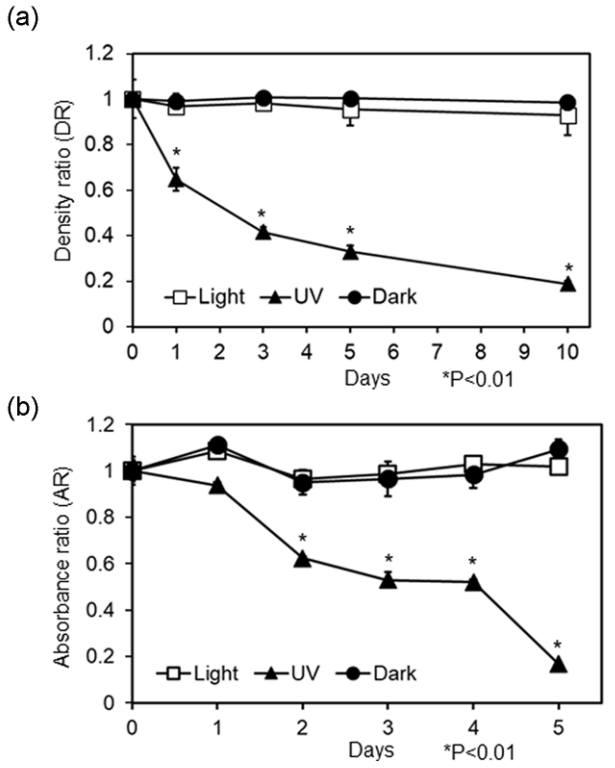


Figure 7. Photostability of BY2-melanin. (a) Filter papers spotted with 1 µL of BY2-melanin dissolved in 0.5 M NaOH at a concentration of 10 mg/mL were exposed to a solar light lamp (60 W, white squares), a UV lamp (253 nm, black triangles), or placed in the dark (black circles) at 25 °C. After 1, 3, 5, and 10 days, images of the BY2-melanin spots on the filter paper were captured with an optical scanner, and their densities were quantified with ImageJ. The ratio of the initial density to the density at each measurement (DR) was calculated to indicate the stability (fading). (b) The BY2-melanin dissolved in 0.5 M Tris–HCl (pH 8.0) at a concentration of 0.05 mg/mL was placed in small glass Petri dishes. These were exposed to a solar light lamp (60 W, white squares), a UV lamp (253 nm, black triangles), or placed in the dark (black circles) at 25 °C for 5 days. The absorbance (λ_{218}) was measured daily and ratioed to the starting absorbance to indicate the stability (denaturation) as the AR. Data points represent the mean (markers) ± standard deviation (bars), acquired in triplicate, and statistical significance was determined by paired *t*-tests. *$P < .01$. All data are representative of three independent experiments.

Suppression of blackish-browning of BY-2 cells by tyrosinase inhibitors

The presence of nitrogen suggests that BY2-melanin is not allomelanin but an eumelanin-like substance, suggesting the involvement of tyrosinase-mediated oxidation of tyrosine or L-DOPA to L-DOPAquinone in the initiation process of BY2-melanin synthesis. To clarify the involvement of tyrosinase, it was examined whether tyrosinase inhibitors would suppress the blackish-browning of the BY-2 cells for up to 2 weeks after transfer. Two well-known tyrosinase inhibitors, KA and PTU, were used in this test (Zolghadri *et al.* 2019).

First, it was examined whether each inhibitor could prevent the growth of BY-2 cells. Immediately after the transfer of BY-2 cells, KA and PTU were administered to the cultures at concentrations of 0.05–2.5 mM and 0.02–1.0 mM, respectively, and the growth of the BY-2 cells was examined. As a result, 0.5 mM of KA slightly suppressed the growth of BY-2, but 2.5 mM significantly suppressed it (Figure S9a, KA). On the other hand, when PTU was 0.02 mM, a weak growth suppression was observed, and the suppression became strong in a dose-dependent manner (Figure S9a, PTU). These results indicated that administration of these inhibitors at the start of culture was unsuitable for observation of suppression of blackish-browning.

Next, since the accumulation of BY2-melanin occurs around 10 days after transfer, KA and PTU were administered at 0.5 and 2.5 mM and 0.02 and 0.1 mM, respectively, 7 days after transfer, when BY-2 cell growth almost completes (stationary phase). Blackish-browning of the cells was observed for the next 7 days after administration (2 weeks after transfer). As a result, a suppression of blackish-browning at the flask level was not observed well at both concentrations of KA (Figure S9b), but a more detailed observation in a test tube and on filter paper revealed that these cells were slightly paler and brownish in color compared to the untreated cells (Figure S9c). On the other hand, the administration of PTU clearly suppressed the blackish-browning of cells even at the flask level (Figure 10a). For further confirmation, a comparison of cells in the test tube and on filter paper clearly showed the suppression of blackish-browning but was not dependent on the concentration of PTU administered (Figure 10b).

From the above, among the tyrosinase inhibitors, PTU clearly suppressed the blackish-browning of BY-2 cells, and KA also showed a weak suppression effect, suggesting the involvement of a tyrosinase-like enzyme in the BY2-melanin synthesis.

Discussion

In plant tissue culture, it is empirically known that brown to black substances accumulate in the cells during culture, and most of these substances are thought to be oxides of phenolic substances (Bhat and Chandel 1991; Jones and Saxena 2013). Despite the description that melanin is included in the brown-black substances (Kaur *et al.* 2018), it seems that melanin has not been identified as a causal substance. The tobacco (*N. tabacum*) cell line BY-2 is one of the most used plant cell cultures worldwide (Nagata, Nemoto and Hasezawa 1992; Nagata, Sakamoto and Shimizu 2004). Researchers using BY-2 cells are well aware of the phenomenon of browning or blackening when these cells enter the decline phase after long-term culture or when the cells are damaged. However, there seem to be no reports on the causative substance so far. In the present study, the substance involved in the browning to blackening of BY-2 cells was a melanin-like pigment, strongly supported by many physicochemical features, such as solubility in alkaline solutions but not a high concentration of HCl and water, and discoloration by an oxidizing agents (Selvakumar *et al.* 2008; Zhan *et al.* 2011).

The BY2-melanin was stable at a high temperature (100 °C) and was resistant to solar lamp irradiation but sensitive to UV irradiation and oxidants, resulting in color fading. It was well soluble in alkaline solutions such as NaOH, KOH, and NH_4OH, but only slightly soluble in low-concentration HCl (0.1 M). In addition, it was not soluble in most organic solvents but was highly soluble in neutral to weakly alkaline buffer solutions. These physicochemical characteristics are similar to those of general melanin. This is the first report that tobacco plants, as well as BY-2 cells, accumulate melanin-like pigments.

The ESR parameters of BY2-melanin were close to those of the reference melanin powders, as shown in Table 2 (Mason, Ingram and Allen 1960; El-Obeid *et al.* 2006; Yao, Qi and Wang 2012; Varga *et al.* 2016), suggesting that BY2-melanin can be assigned to a melanin analog. This assignment follows other spectroscopic results shown in this study. To put it more precisely, the BY2-melanin has a *g*-value almost identical to that of Sepia melanin for which the isotropic *g*-value is 2.0035. As the *g*-value is a probe sensitive to the electronic structure where an

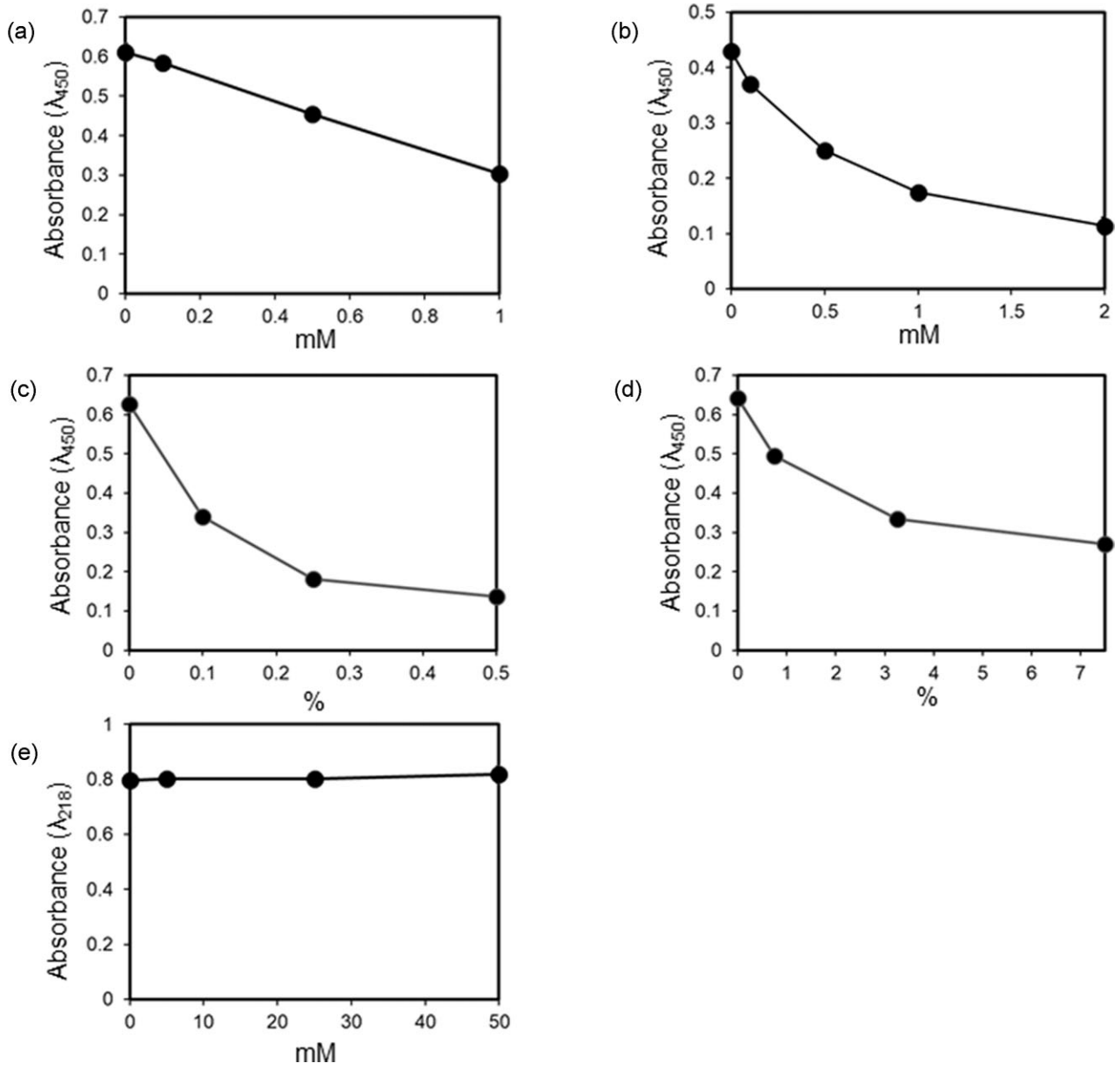


Figure 8. Reaction to various oxidizing and reducing agents. The concentration of BY2-melanin was 0.5 mg/mL (a) BY2-melanin dissolved in 0.5 M Tris–HCl (pH 8.0) was added to 0–1 mM of $KMnO_4$ and left at room temperature for 2 h in the dark. (b) BY2-melanin dissolved in 0.1 M HCl was added to 0–2 mM of $K_2Cr_2O_7$ and left at room temperature for 24 h in the dark. (c) BY2-melanin dissolved in 0.5 M NaOH was added to 0%–0.5% NaOCl and left at room temperature for 2 h in the dark. (d) BY2-melanin dissolved in 0.5 M NaOH was added to 0%–7.5% H_2O_2 and left at room temperature for 2 h in the dark. (e) BY2-melanin dissolved in 0.5 M NaOH was added to 0–50 mM of Na_2SO_3 and left at room temperature for 2 h in the dark. The absorbance was measured at a wavelength of 450 nm (λ_{450}) in (a–d) and 218 nm (λ_{218}) in (e). Data points represent the mean (markers) ± standard deviation (bars) acquired in triplicate. All data are representative of three independent experiments.

unpaired electron is distributed, the structure of the radical in BY2-melanin is probably similar to that in the Sepia melanin rather than synthetic melanin. On the other hand, BY-2 showed no hyperfine splitting, similar to Sepia and synthetic melanins. Since the specific structure as a macromolecule of BY-2 is currently unknown, there is no meaning to discuss the radical structure. However, it may be rational to presume a formation of semiquinoid-type radicals, which give a single line of ESR with no hyperfine splitting, stabilized through C=C conjugations in BY-2 as that reported for Sepia melanin (Mason, Ingram and Allen 1960).

The ΔH_{pp} of BY2-melanin was larger than that of the two reference melanins (Table 2). Assuming that the difference in the linewidth is caused by electron spin relaxations, the BY2-melanin may have a stronger interaction with the surroundings compared with the two reference melanins. A stronger interaction of the electron spin with its surroundings should shorten the relaxation time, increasing the linewidth in ESR. The radical content per unit weight of BY-2 was the smallest of the three melanins. In the previous report, the chemical reduction of Sepia melanin with ascorbic acid resulted in loss of color and simultaneous decrease in free radical content (Mason, Ingram

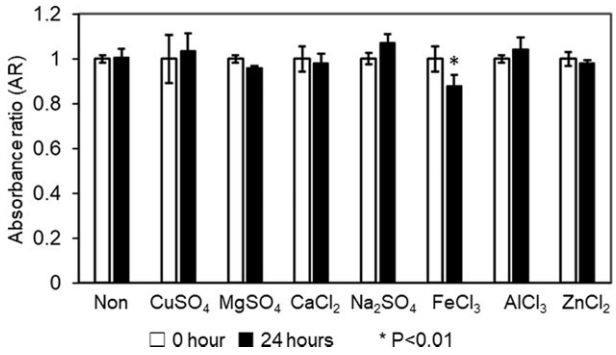


Figure 9. Reaction to metal ions. $CuSO_4$, $MgSO_4$, $CaCl_2$, Na_2SO_4, $FeCl_3$, $AlCl_3$, or $ZnCl_2$ were added to a 0.05 mg/mL BY2-melanin solution in 0.1 M NaOH, to a concentration of 0.1 mM, and the absorbance (λ_{218}) was measured after 0 and 24 h. "Non" indicates a control containing no metal ion compounds. Taking the absorbance at 0 h as 1, the AR was calculated from the absorbance at 24 h. Data points represent the mean ± standard deviation (thin black bars) acquired in triplicate, and statistical significance was determined by paired *t*-tests. *P < .01. All data are representative of three independent experiments.

and Allen 1960). The smallest radical content in BY-2 may be related to its light-blackish color.

The most significant finding in our study is that BY2-melanin is a novel plant-eumelanin-like pigment distinct from that of plants and other organisms. The constituent elements of BY2-melanin were C, H, O, and N, with an N content of ~10% and a C/N molar ratio of 5.95 (Table 1). Most of the melanin in plants is allomelanin, the precursors of which include catechol, DHN, caffeic acid, chlorogenic acid, protocatechuic acid, and gallic acid, and which are called catechol melanin (Solano 2014), and nitrogen is not contained or is contained in a small amount (d'Ischia *et al.* 2015). Indeed, the N content is ~1% in chestnut shell melanin (Yao, Qi and Wang 2012), ~3% in tea leaf melanin (Sava *et al.* 2001), and ~1.7% in sunflower seed coat melanin (Li, Chen and Tang 2018), and their C/N molar ratios are approximately 36–72, 19, and 41, respectively. Therefore, the BY2-melanin was considered to be different from the plant-derived melanins reported previously. On the other hand, since the N content of synthetic melanin (an eumelanin) is ~8.25% and the C/N molar ratio is higher than 7.1, and Sepia melanin (an eumelanin derived from *Sepia*) has a C/N molar ratio of 7.73 (Ito 1986), the BY2-melanin is rather close to these. In addition, since the N content of melanins from the genus *Pleurotus* (mushrooms) was 9.78–11.09% and the C/N molar ratio was 5.57–6.01 (Zhang, Wu and Huang 2022), the BY2-melanin is relatively close to these. FT-IR analysis also supported this view. The BY2-melanin showed a spectrum similar to that of synthetic melanin and Sepia melanin, but the number of peaks was greater, suggesting that this substance has a complex structure (Figure 4). The overall pattern of the FT-IR spectra of BY2-melanin appeared similar to that of mushrooms such as *Lachnum* (Ye *et al.* 2014) and *Pleurotus* (Zhang, Wu and Huang 2022). Zhang, Wu and Huang (2022) deduced that, due to the high nitrogen content and sulfur presence, the oyster mushroom melanin was not allomelanin but a mixture of eumelanin and pheomelanin. Since the presence of sulfur has not been detected in BY2-melanin, it might consist only of eumelanin. This presumption seems to be supported by the results of the suppression of blackish-browning of BY-2 cells using tyrosinase inhibitors (Figures 10 and S9b). In our tyrosinase inhibition test, PTU showed a clear suppression of blackish-browning of BY-2 cells, and KA showed slightly (Figures 10 and S9b). These results suggested that the enzymatic process is involved in BY2-melanin synthesis. To clarify the involvement of tyrosinase, it would be necessary to examine the effects of different types of inhibitors and the timing of their administration. Furthermore, an *in vitro* tyrosinase activity assay using extracts from cells around the blackish-browning stage would be required. On the other hand, many melanins are complex macromolecules in which multiple constituents are tightly

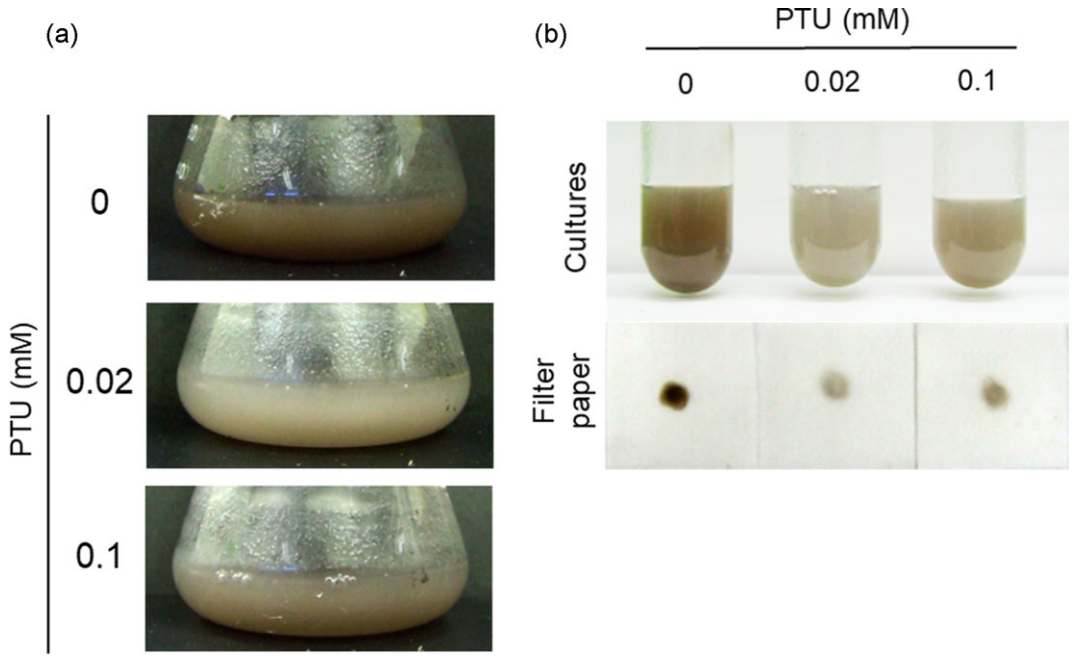


Figure 10. Suppression of blackish-browning of BY-2 cells by tyrosinase inhibitor (PTU). One week after transfer, PTU was administered to final concentrations of 0, 0.02, and 1.0 mM, and the cells were further cultured for 6 days (13 days after transfer). (a) Flasks containing BY-2 cultures with different concentrations of PTU. (b) BY-2 cultures (1 mL each) from the flasks shown in (a) in test tubes (upper), and BY-2 cells after removing the medium from the culture (0.1 mL) shown in (a) with filter paper (lower). The control cells without PTU were blackish-brown, whereas the blackish-browning of cells was clearly suppressed by PTU administration.

bound to each other, making it difficult to determine their structure (Pralea *et al*. 2019). Since BY2-melanin is also robust and is insoluble in organic solvents, we are proceeding with identifying its degradation products obtained by alkali oxidation via liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Melanin accumulates in the hair and skin of humans and other vertebrates, but is also found in nerve cells. In addition to vertebrates, melanin is also found in other species as in the case of cephalopod ink (Derby 2014), bivalve shells (Affenzeller *et al*. 2019), and arthropod epidermis (Barek *et al*. 2018), and is thought to play a role not only in protecting against ultraviolet rays but also in hardening the epidermis to protect against external predators and the environment. It is also found in microorganisms such as fungi, bacteria, and mushrooms and is presumed to be involved in protection from oxidative stress and electron transfer (Tran-Ly *et al*. 2020). In a wide range of plant species, melanin accumulates not only in seed coats but also in leaves and appears to provide resistance to insect damage and drought (Solano 2014; d'Ischia *et al*. 2015). On the other hand, as an industrial use of melanin, squid ink is widely used as a natural black pigment for food (Derby 2014). In particular, there may be little reluctance to use physiologically active plant-derived melanin-like pigments as food additives. In addition, its wide-wavelength UV-Vis absorption function is used as a solar screen in cosmetics and sunglasses (Tran-Ly *et al*. 2020). Furthermore, due to its unique electron transfer properties, it is proposed as an excellent electronic material, such as for batteries and electrodes (Paulin and Graeff 2021). In addition to being used in foods and cosmetics as a substance with antioxidant properties, antiallergic, anti-inflammatory and antitumor effects have also been reported, suggesting the potential for use in pharmaceuticals (Mimura *et al*. 1987; El-Obeid *et al*. 2006; Oberg *et al*. 2009; Kawamoto *et al*. 2019). Evaluation of the UV-protective function and antioxidant activity of BY2-melanin is in progress.

As in most plants, melanin is contained in the seed coat, and its extract material can only be obtained during a particular season of the year. In addition, the quality of the extracted material varies depending on the weather and the place of cultivation. Furthermore, a pulverization process may be necessary to extract the hard seed coats peeled off from the seeds. Therefore, if plant melanin is to be used constantly, a stable supply of extraction materials is required. Plant tissue culture may be a convenient method for that purpose. Regarding the production of melanin-like pigments in plant tissue culture, successful examples have been reported in the cultured cells of Nigella (Haseeb and Elhag 2012). However, this production requires a long-term culture of 8 weeks. Since we clarified that BY-2 cells, which grow the fastest among the established plant-cultured cell lines, can produce eumelanin-like pigments, this cell line is expected to be an excellent material to solve the problem of shortening the culture period. BY-2 cells have been distributed worldwide since the 1980s (Nagata, Nemoto and Hasezawa 1992). Therefore, it is possible that the traits (growth rate, cell mass shape, cell culture color, and so on) gradually changed during long-term culturing in each laboratory, including the cells used in this study. On the other hand, the BY-2 cell line deposited at RIKEN BRC can be considered a standard line of BY-2 cells. A similar blackish-brown discoloration occurred when this standard cell line was cultured under the conditions shown in this study (unpublished results). This result suggested that this blackish-browning property of BY-2 cells after long-term culture is not only exhibited by the line of our laboratory but was originally present in the BY-2 cell line obtained at the Japan Tobacco and Salt Public Corporation.

In the present study, we established a method for the successful accumulation and isolation of eumelanin-like pigment from BY-2 cells, cultured for 3 weeks in a small flask with a reduced medium volume. However, the appropriate medium and conditions that allow efficient accumulation of BY2-melanin in a short culture period remain to be investigated.

Conclusion

The present study revealed that tobacco BY-2 cells in a long-term liquid culture accumulated melanin-like pigment inside the cells, which subsequently took on a blackish-brown color. Some of the pigments outflowed into the medium, and thus the medium exhibited a blackish-brown color. We successfully isolated blackish-brown amorphous particles (BY2-melanin) from the cultured BY-2 cells by elution with NaOH and precipitation with HCl, followed by treatment with organic solvents. SEM, elemental composition, UV-Vis spectrum, FT-IR, and ESR analyses revealed that the characteristics of this pigment were similar to those of eumelanin. The physicochemical properties of the isolated BY2-melanin make it a promising candidate for novel plant-derived eumelanin, and the tyrosinase inhibition test also supported this possibility. Therefore, this study has developed a suitable protocol for the commercial production of plant-derived eumelanin in a relatively short time and at a low cost that can easily replace the other conventional melanin production processes. An area for further research may be to evaluate the various biological activities of BY2-melanin, such as UV protection and antioxidation activity, and other health-beneficial effects, such as antiallergic and anticancer activity, for pharmaceutical drug discovery.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

A.T.M.R.I. and N.T. made the conception and design of the work, carried out experiments, and drafted the original manuscript. K.S. and H.M. carried out the FT-IR analysis. K.Kom. carried out the ESR analysis. K.Koi. carried out the SEM observations. K.A. and K.Ki. made substantial contributions to the conception of the work. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

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