Melanin decolorization by lysosome-related extract in *Saccharomyces cerevisiae* modified to overproduce glutathione peroxidase

Gyeongchan Jeon[1] · Younga Kim[2] · Su Yeon Choi[1] · Yang-Hoon Kim[3] · Jiho Min[1]

Abstract

All eukaryotes have lysosomes that contain hydrolytic enzymes, such as protease, that degrade waste materials and cellular fragments. As a cellular organelle, lysosomes function as the digestive system of the cell, serving both to degrade material taken up from outside the cell and to digest obsolete components of the cell itself. In a previous study, melanin compounds were bleached using lysosome-related organelle extract (LOE) in which glutathione peroxidase (GPX) contributed decisively to melanin decolorization. In this study, *Saccharomyces cerevisiae* was engineered to overproduce GPX, which increases the melanin color reduction activity of LOE. In addition, the peroxidase activity of the recombinant yeast was measured for each compartment. In spite of the modification to overexpress the GPX protein, with the peroxidase activity of the lysosome fraction specifically higher, the overall peroxidase activity of the cells remained constant. The overexpression of GPX2 among the GPX present in *S. cerevisiae* increased both the melanin-decolorization activity and the peroxidase activity of LOE. These results indicate that the peroxidase activity is related to the melanin decomposition and antioxidant enzymes such as GPX. In an artificial skin tissue test, the LOE extracted from the recombinant yeast was efficient in reducing the melanin. These results confirmed the enzyme's ability to penetrate corneous tissue, and they suggest the possibility of further development as a new whitening cosmetic.

Key points
- *Modification of Saccharomyces cerevisiae to overexpress glutathione peroxidase (GPX).*
- *The lysosome fraction of the recombinant strain enhanced the decolorizing function.*
- *The LOE penetrates the skin barrier and works effectively on artificial skin tissue.*

Keywords Melanin · Glutathione peroxidase (GPX) · *Saccharomyces cerevisiae* · Lysosome

Introduction

Hyperpigmentation that occurs on the face or back is a major headache for the clinical beauty industry. Pigmentation is related to UV exposure, skin aging, and genetic factors, and it is caused by an overproduction of melanin compounds in the skin (Ichihashi and Ando 2014). The melanin compound is synthesized by tyrosine oxidation in melanocyte cells in the dermis, and it is transferred to the keratinocytes, which are found in the basal layer of the epidermis (Mason 1948). The primary role of this pigment in the skin is to protect the dermis by blocking harmful ultraviolet radiation, so melanin compounds are essential for life under the sun, but abnormally produced melanin causes problems such as hyperpigmentation (Korać and Khambholja 2011). Currently, skin whitening cosmetics have a function to inhibit melanin

✉ Yang-Hoon Kim
  kyh@chungbuk.ac.kr

✉ Jiho Min
  jihomin@jbnu.ac.kr

1 Graduate School of Semiconductor and Chemical Engineering, Jeonbuk National University, 567 Baekje-daero, Deokjin-gu, Jeollabuk-do 54896, Jeonju-si, Republic of Korea

2 Department of Bioprocess Engineering, Jeonbuk National University, 567 Baekje-daero, Deokjin-gu, Jeollabuk-do 54896, Jeonju-si, Republic of Korea

3 Department of Microbiology, Chungbuk National University, Chungdae-Ro, Seowon-Gu, Cheongju 28644, South Korea

synthesis. However, these products take a long time to take effect and are not adequate to treat hyperpigmentation, such as melasma. Following the recent increase in aesthetic interests, much research is underway regarding materials and therapies to decompose overproduced melanin compounds.

Previously, we found that lysosome-related organelle extract (LOE) was effective to reduce melanin color, and the melanin decolorization efficiency was related to the peroxidase activity of the enzymes (Yoon et al. 2015; Park et al. 2020). Lysosome-related organelle includes several functional cellular organelles, including lysosomes and peroxisomes. Lysosomes are cellular organelles that contain acidic enzymes to break down waste materials and cellular debris in all eukaryotes (Yoon et al. 2009). Lysosomes were reported to contain about 50–60 enzymes, including several hydrolytic enzymes, and they play an essential role in several biological processes (Anderson and Parrish 1981; Ni et al. 2006). The degradation pathway of the lysosomes generally takes place via endocytosis, which provides the extracellular materials for digestion (Česen et al. 2012). The peroxisomes or microbodies are limited membrane vesicles like the lysosomes. Unlike lysosomes, peroxisomes are pinched off from the endoplasmic reticulum and not from the Golgi apparatus. Peroxisomes contain some oxidative enzymes such as catalase, urate oxidase, and Damino acid oxidase. The peroxisomes generate hydrogen peroxide and scavenge the activities of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bonekamp et al. 2009; Islinger et al. 2018). Additional studies have been conducted to determine which of the enzymes contribute to the degradation of melanin in lysosomes and peroxisomes. We investigated glutathione peroxidase (GPX), an antioxidant enzyme in LOE, for how it accelerates melanin decoloration in the presence of hydrogen peroxide and inhibits melanin synthesis in B16F10 melanoma cells (Jeon et al. 2021). GPX is located at the peroxisome and cytoplasm, and it contributes to the antioxidant activity of the cells by catalyzing the conversion of hydrogen peroxide, a highly reactive substance, into water, a stable substance (Bhabak and Mugesh 2010; Ohdate and Inoue 2012).

In this study, we analyze the intracellular effect of GPX by modifying *Saccharomyces cerevisiae* to overproduce GPX. There are three types of GPXs in yeast, GPX1, GPX2, and GPX3. Therefore, three strains were engineered to insert the responsible genes into a plasmid to synthesize. The expression of GPX was confirmed via a tagged fluorescent protein in the recombinant yeast. The peroxidase activity of LOE and the whole protein extracted from the clones was compared, and the effect of the GPX produced in the intracellular organelles was analyzed. The peroxidase activity was measured by pyrogallol oxidation. The most commonly used substrates for phenol oxidase and peroxidase assays in the soil are pyrogallol (1,2,3-trihydroxybenzene), DOPA (l-3,4-dihydroxyphenylalanine), and ABTS (2,2′-azino-bis

(3-ethylbenzthiazoline-6-sulfonic acid)) (Bach et al. 2013). In order to confirm the effect of the GPX in yeast on the melanin decomposition activity, the LOE extracted from the recombinant strains was treated with melanin and was compared to the control strain. In addition, we examined the whitening ability of the LOE in artificial human skin tissue to verify the whitening effect in the actual skin. The LOE was treated into artificial tissue over 16 days to evaluate the melanin reduction.

In conclusion, the modified yeast strains were more useful to reduce the melanin color than the control strain, and the peroxidase activity of the clones was higher than that for control. The melanin bleaching efficiency was better in the order of GPX2, GPX1, GPX3, and the peroxidase activity showed the same tendency. Furthermore, in the artificial tissue test, the LOE isolated from the modified yeast strains was effective in reducing the melanin color, and the results were similar to those of the melanin bleaching experiment. However, despite the amplification of GPX, the overall peroxidase activity of the cells remained constant. In this study, by amplifying GPX in *S. cerevisiae*, the melanin-decolorization efficiency of LOE improved, and we found the enzyme had the ability to penetrate corneous tissue in the skin by conducting an artificial tissue test. The relationship between the melanin decomposition activity and the peroxidase activity was revealed, and further studies are needed on the peroxidase activity and melanin decomposition in the cells. This is the first study to apply GPX, an antioxidant enzyme, and the lysosome fraction of yeast to whitening functional cosmetics.

Materials and methods

Strains and plasmids

All *S. cerevisiae* and *Escherichia coli* strains used in this study are 2805 (ATCC 208,280) and DH5α (RBC RH617), respectively. The strains, plasmid, and PCR primers used are summarized in Tables 1 and 2.

First, to clone the *GPX* genes into yeast, plasmids containing the *GPX* gene were produced. All *GPX* genes were replicated from the genomic DNA of the *S. cerevisiae* strain 2805. After that, the expression of a GPX protein was confirmed by fusion with a fluorescent protein, cyan fluorescent protein (CFP). We used the pYES2.0 vector as the backbone vector and isolated the chromosomal DNA from *S. cerevisiae* by using a Genomic DNA Isolation Kit (CMX0112, LaboPass, Seoul, Republic of Korea). The three *GPX*s present in the yeast genome were amplified via PCR using respective primers (Table 2) and the chromosomal DNA of *S. cerevisiae*: glutathione peroxidase 1 gene (*GPX1*, GenBank: AAS56221), glutathione peroxidase 2 gene (*GPX2*, GenBank: AAS55967), and glutathione peroxidase 3 gene (*GPX3*, NCBI Reference Sequence: NP_012303). The PCR fragments

Table 1 Strains and plasmids used in this study

Strain	Genotype	Reference
Escherichia coli strain		
DH5α	*F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44-thi-1 gyrA96 relA1*	RBC (Banqiao, Taiwan)
Saccharomyces cerevisiae strains		
2805	*MATα pep4::HIS3 prb1-Δ1.6R can1 GAL2 his3-Δ200 ura3-52*	ATCC (Manassas, USA)
MBTL-GC0	2805, pYES2.0 (Mock vector)	This study
MBTL-GC1	2805, pYES2.0::*GPX1*::*CFP*	This study
MBTL-GC2	2805, pYES2.0::*GPX2*::*CFP*	This study
MBTL-GC3	2805, pYES2.0::*GPX3*::*CFP*	This study
Plasmid	Description	Reference
pECFP	pLP-*ECFP*-C1	Clontech Laboratories (CA, USA)
pYES2.0	*URA3* (control plasmid)	Invitrogen (MA, USA)
pYES2.0::GPX1::CFP	*URA3*, expression of *GPX1* gene and *CFP* gene	This study
pYES2.0::GPX2::CFP	*URA3*, expression of *GPX2* gene and *CFP* gene	This study
pYES2.0::GPX3::CFP	*URA3*, expression of *GPX3* gene and *CFP* gene	This study

Table 2 Primers for amplification of GPX gene in this study

Primer	Sequence	Purpose
GPX1-F	GGCAAGCTTATGCAAGAATTTTA	Forward primer for pYES2.0::GPX1
GPX1-R	TGAGGATCCAATCTGTTCTTCTGG	Reverse primer for pYES2.0::GPX1
GPX2-F	GGCAAGCTTATGACCACATCTTTTTATG	Forward primer for pYES2.0::GPX2
GPX2-R	GCCGGATCCTTTACTTAACAGGCTTTG	Reverse primer for pYES2.0::GPX2
GPX3-F	GGCAAGCTTATGTCAGAATTCTATAA	Forward primer for pYES2.0::GPX3
GPX3-R	ACTGGATCCTTCCACCTCTTTCAA	Reverse primer for pYES2.0::GPX3
CFP-F	ATTGGATCCGTGAGCAAGGGCGAG	Forward primer for pYES2.0::GPX1::CFP, pYES2.0::GPX2::CFP, pYES2.0::GPX3::CFP
CFP-R	GCCTCTAGATTACTTGTACAGCTCGTCC	Reverse primer for pYES2.0::GPX1::CFP, pYES2.0::GPX2::CFP, pYES2.0::GPX3::CFP

were inserted into the *Hin*dIII and *Bam*HI sites of the pYES2.0 vector to make pYES2.0::*GPX1*, pYES2.0::*GPX2*, pYES2.0::*GPX3* plasmids. Then, the *CFP* gene was amplified from pECFP by PCR with primers CFP-F and CFP-R. The PCR product was cloned into the *Bam*HI and *Xba*I sites of pYES2.0::*GPX1*, pYES2.0::*GPX2*, pYES2.0::*GPX3* to produce pYES2.0::*GPX1*::*CFP* (Fig. 1), pYES2.0::*GPX2*::*CFP*, and pYES2.0::*GPX3*::*CFP* (Fig. 1). All recombinant plasmids were inserted into *E. coli* for amplification; then, the sequence was analyzed by electrophoresis and Sanger sequencing. The

Sanger sequencing was performed with an Applied Biosystems SeqStudio Genetic Analyzer, ABI3730XL (Bioneer corp, Daejeon, Republic of Korea). Then, we analyzed the sequence similarity by using the Basic Local Alignment Search Tool (BLAST) of the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi, MD, USA). The PCR products were purified using an Expin PCR SV kit (GeneAll, Seoul, Republic of Korea), and the plasmids inserted into *E. coli* were isolated using the Expin Plasmid SV kit (GeneAll, Seoul, Republic of Korea).
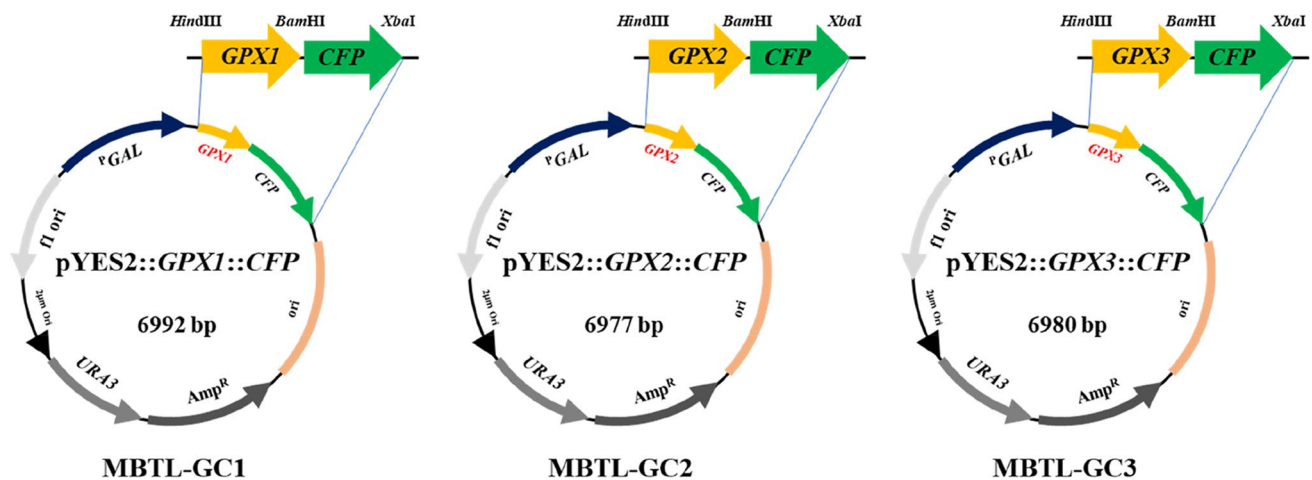
Fig. 1 Structures of the recombinant plasmids of (**a**) MBTL-GC1, (**b**) MBTL-GC2, and (**c**) MBTL-GC3

Transformation

All produced plasmids were inserted into *E. coli* via electroporation and transformants were selected by using Luria-Bertoni (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCL) with 50 μg/mL ampicillin. After culturing at 37 °C in liquid LB-amp medium, plasmids were isolated. After that, they were transformed into *S. cerevisiae* to generate recombinant *S. cerevisiae* MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3 (Table 1). The *S. cerevisiae* transformations were performed using the lithium acetate method (Schiestl and Gietz 1989).

Confirmation of modified GPX protein expression and culture condition

Mock and recombinant *S. cerevisiae*, MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3, were grown in synthetic dropout (SD) medium (0.67% yeast nitrogen base, 0.5% casamino acid, 2% D-glucose) at 30 °C with shaking at 180 rpm for 20 h. To induce the *GAL1* promoter of the pYES2.0 vector, galactose was added at a concentration of 2%. After being added, the cells were incubated at 30 °C with shaking at 180 rpm for 20 h to grow until the log-phase. Then, they were harvested by centrifugation at 3000 rpm for 5 min. After discarding the supernatant, the cells were washed with sterilized distilled water, then observed with a confocal laser-scanning microscope (CLSM) to confirm the presence of CFP-tagged protein ($\lambda_{Excitation}$: 435 mm, $\lambda_{Emission}$: 485 mm). All samples were analyzed by the CLSM (LSM 880 with Airyscan, Carl Zeiss, Oberkochen, Germany) installed in the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University.

LOE isolation

Lysosomes were isolated from mock and recombinant *S. cerevisiae* using cell fractionation (Yoon et al. 2009, 2010).

To extract lysosomes from recombinant yeast strains, they were cultured under the previously mentioned culture conditions and were then harvested. To remove the cell wall thoroughly, 5 g of the cells was mixed with the buffer containing 100 mM Tris-SO$_4$ (pH 9.4) and 10 mM 1,4-dithiothreitol (DTT), then was incubated at 30 °C in a shaking incubator at 90 rpm for 15 min. The mixture was suspended, then centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. Then, 5 g of the cells was re-suspended in breaking buffer (20 mM Tris–Cl (pH 7.4), 0.6 mM sorbitol, 1 mM phenylmethylsulfonyl fluoride (PMSF)) to relax the membranes of the cells. Glass beads were added to these cells at a mass ratio of 1:1. Then, the mixtures were repeatedly vortexed and kept on ice at an interval of 1 min for a total of 20 min. After 10 min of stabilization on ice, centrifugation was carried out at 500 g for 5 min. The supernatant was collected in a microtube and then centrifuged at 20,000 g for 30 min at 4 °C. Finally, 0.5 g of lysosome was obtained. LOE was extracted with a lysis buffer (0.1% Tergitol-type NP-40, 5 mM DTT, and 0.1 mM PMSF) at a volume ratio of 1:1. The mixture was then vortexed for 10 min, followed by a 30-min reaction time on ice. After homogenization, the mixture was centrifuged at 13,000 rpm for 10 min at 4 °C, and the fresh enzyme in the supernatant content was used for each assay.

Cell lysis

The whole protein of the recombinant yeasts, MBTL-GC1, MBTL-GC2, MBTL-GC3, and MBTL-GC0, was extracted via cell lysis.

Two milligrams of the cells was mixed with a lysis buffer (0.1% NP-40, 5 mM DTT, and 0.1 mM PMSF) at a volume ratio of 1:1 and was then homogenized via sonication. The cells suspended in the lysis buffer were sonicated with a short burst of 20 s followed by intervals of 60 s for cooling. The sonication process was repeated 10 times, and all operations were performed at 0 to 4 °C. The homogenized cells were then centrifuged at 20,000 g for 20 min at 4 °C, and the whole protein in the supernatant content was used for each assay.

Peroxidase activity assay

Peroxidase assays were performed following the Britton Chance method (Chance and Maehly 1955; Serra et al. 1992). The peroxidase activity of the LOE and whole protein was measured as pyrogallol oxidation at 420 nm in 100 mM Tris buffer, pH 7.0.

Four types of LOE and whole protein were isolated from each recombinant yeast under the previously mentioned culture conditions and extraction methods. Thirty microliters of each sample was mixed with 170 μL of assay buffer (100 mM Tris–HCl, 0.5 mM pyrogallol, 0.02 mM H_2O_2) in a 96-well plate, and the absorbance was measured at 420 nm for 10 min at 2-min intervals. The peroxidase activities were calculated as estimated from the slope of the progress curves and were expressed as follows: 1 Unit = the increase in 0.001 absorbance at 420 nm per min. All assays were performed at 25 °C, and the absorbance was measured using microplate spectrophotometry.

Melanin color reduction

The melanin solution contains 0.1 mM PBS (phosphate buffered saline, pH: 7.0) and melanin powder (synthetic, Sigma, St. Louis, USA) that was prepared via oxidation of tyrosine with hydrogen peroxide. One hundred parts per million of melanin solution was mixed with 10% (v/v) LOE and was reacted for 10 days at room temperature. The four types of LOE that were used were extracted from mock, MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3, in the same way. The melanin content was measured by absorbance at 450 nm. To determine the melanin concentration, a standard curve was prepared from an authentic standard of synthetic melanin (R2 = 0.999, data not shown). The melanin decolorization value was determined by subtracting the melanin concentration after the reaction, from the initial value.

Artificial tissue culture condition

For the whitening tests in skin tissue, MelanoDerm (Mat-Tek Corp, Ashland, MA) was used as a viable reconstituted three-dimensional human skin equivalent containing normal, human-derived epidermal keratinocytes (NHEK), and melanocytes (NHM) that have been cultured to form a multilayered structure. The cells are derived from African-American (MEL-B), Asian (MEL-A), or white (MEL-C) donors. In this study, the MEL-B tissues were maintained in NMM-113 medium — a serum-free medium without artificial stimulators of melanogenesis such as 12-O-tetradecanoylphorbol 13-acetate (TPA) and isobutylmethylxanthine (IBMX) (Fischer et al. 2008; Tardy et al. 2006). The MelanoDerm tissues were fed every other day with 5 mL of fresh NMM-113 and 10% LOE isolated from recombinant strains every other day for 16 days. Additionally, 1% kojic acid was used as positive control.

Melanin content assay in artificial tissue

The melanin content was measured following previously reported protocols (Ni-Komatsu et al. 2008). After 16 days of culture, the skin tissue was taken in an Eppendorf tube for homogenization. The tissues were sonicated for 1 min in 450 μL of lysis buffer (1% (v/v) sodium dodecyl sulfate (SDS), 50 μM ethylene-diamine-tetraacetic acid (EDTA), 10 mM Tris–HCl (pH: 6.8)). After homogenization, 20 μL protease K (5 mg/mL) was added and incubated at 45 °C overnight. Twenty microliters of protease K was placed into the sample the next day for 4 h at room temperature. Fifty microliters of 500 mM sodium carbonate and 10 μL of 30% H_2O_2 were added and incubated at 80 °C for 30 min. Then, 100 μL of a 2:1 chloroform:methanol mixture was added. The mixture containing significantly homogenized tissue was centrifuged at 10,000 × g for 10 min; then, the supernatant was used to determine the melanin content using a spectrophotometer based on the absorbance at 450 nm.

Histological analysis

To visualize the melanin distribution throughout the tissue, the keratinocyte and melanocyte of the tissue were stained with hematoxylin and eosin (H & E). The hematoxylin stains the cell nuclei blue, and the eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades, hues, and combinations of these colors (Bancroft and Gamble 2008; Chan 2014). This analysis was performed following previously published protocols (Ni-Komatsu et al. 2008; Yoon et al. 2011). After 16 days of culture, the skin tissues were fixed with 10% of formalin overnight; then, paraffin infiltration and H & E staining proceeded in order. The samples were observed under phase-contrast microscopy. All paraffin-embedded tissue samples were processed as previously described to visualize the melanin distribution throughout the tissue, and the tissue slices were observed under phase-contrast microscopy.

Results

Construction of recombinant *S. cerevisiae*

The vectors containing GPX genes were confirmed via electrophoresis and DNA sequence analysis and then inserted into *S. cerevisiae* to overexpress GPX proteins. The vectors contained *GPX1*, *GPX2*, and *GPX3* gene present in *S. cerevisiae*, respectively, and *CFP* gene was attached to confirm the expression of a GPX in the cells. Recombinant plasmids pYES2.0::*GPX1*::*CFP*, pYES2.0::*GPX2*::*CFP*, and pYES2.0::*GPX3*::*CFP* were successfully transformed into *S. cerevisiae* via electroporation to generate the recombinant *S. cerevisiae* MBTL-GC1,

MBTL-GC2, and MBTL-GC3 (Table 1). The recombinant *S. cerevisiae* MBTL-GC0 was transformed by inserting pYES2.0 and was used as a control. *GPX* was induced by the *GAL1* promoter with SG medium (2% D-galactose) at 30 °C for 20 h. After culturing and induction, the expression of the GPX protein attached with CFP was confirmed via confocal laser scanning microscopy (LSM 880 with Airyscan, Carl Zeiss, Oberkochen, Germany) with an excitation/emission of 458 nm/522 nm for the cyan fluorescent protein. We found the fluorescent signal in recombinant *S. cerevisiae* (Fig. 2).

Fig. 2 Fluorescent images of recombinant *S. cerevisiae*, (**a**) MBTL-GC0, (**b**) MBTL-GC1, (**c**) MBTL-GC2, and (**d**) MBTL-GC3. After culturing and induction, the expression of the GPX protein attached with CFP was confirmed via confocal laser scanning microscopy (LSM 880 with Airyscan, Carl Zeiss, Oberkochen, Germany) with an excitation/emission of 458 nm/522 nm for the cyan fluorescent protein. Items (b), (c), and (d) indicate the GPX1, GPX2, and GPX3 proteins, respectively. Item (a) was the control *S. cerevisiae*, MBTL-GC0
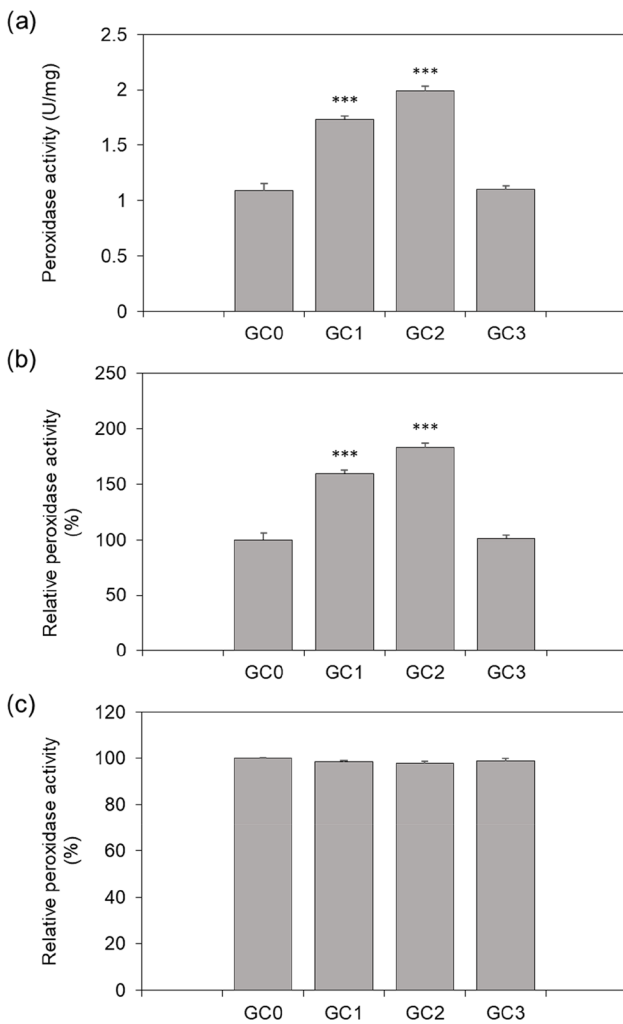
(a)

(b)

(c)
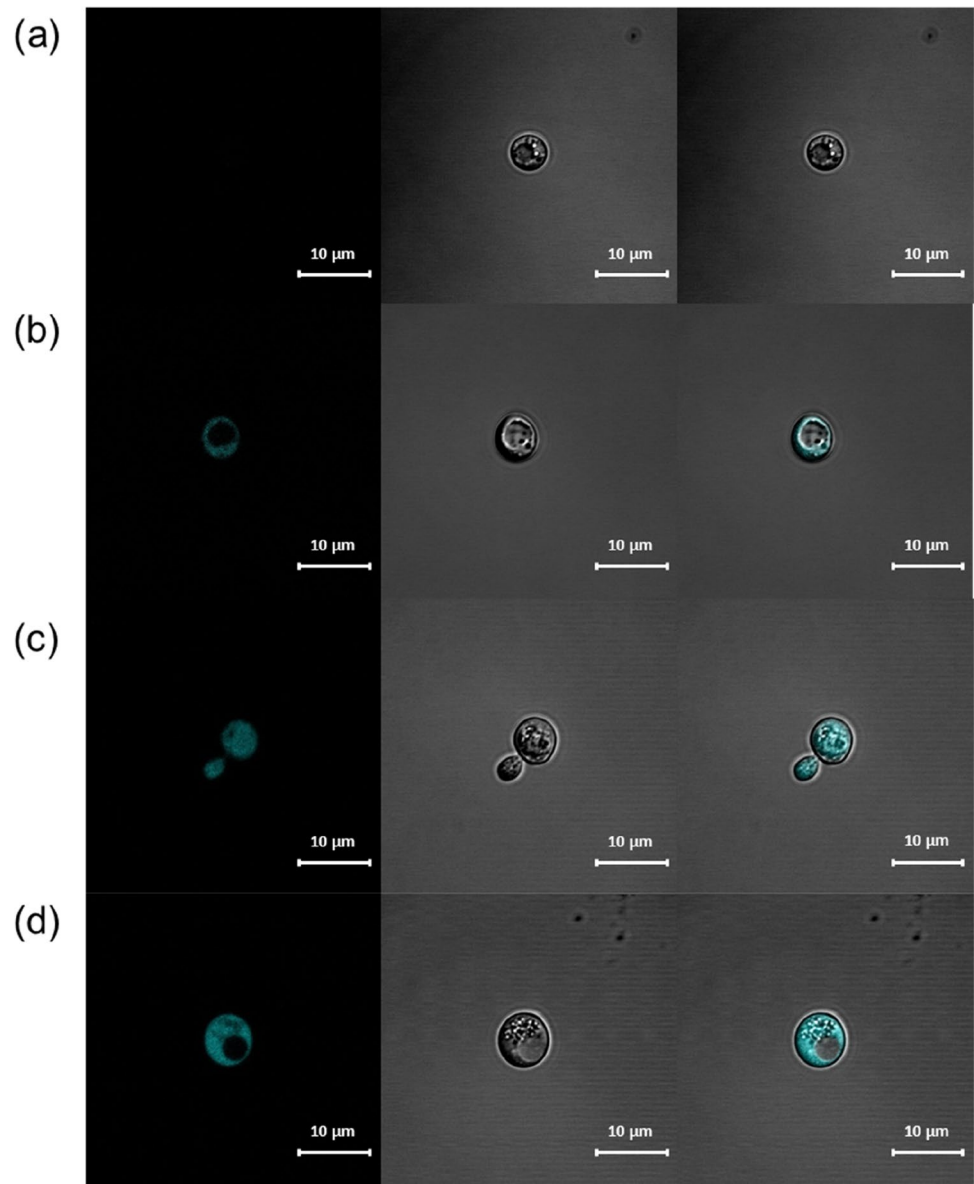
Fig. 3 (**a**) Peroxidase activity of LOE isolated from recombinant *S. cerevisiae*, and relative peroxidase activity of (**b**) LOE and (**c**) whole protein isolated from recombinant *S. cerevisiae*. GC0, GC1, GC2, and GC3 were treated with LOE extracted from MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3, respectively. MBTL-GC0 is the control strain. Data are expressed as a percentage of GC0 and presented as the means ± SD (*n* = 3). ***P < 0.001 vs. GC0
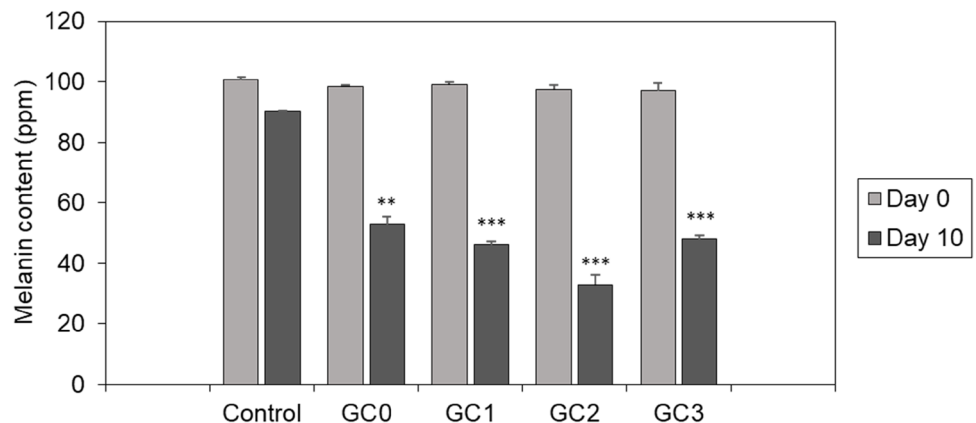
Peroxidase activity assay

This part of the study analyzed the effect of the GPX overexpression on peroxidase activity in the cells and organelles. The peroxidase activity of the whole protein and LOE isolated from the recombinant *S. cerevisiae* was measured and compared. The peroxidase activity of the samples was determined via pyrogallol oxidation in 100 mM Tris buffer, pH 7.0, and the oxidation of pyrogallol was verified by an increase in the absorbance at 420 nm. In this research, 1 unit of peroxidase activity was defined as the increase in the absorbance of 0.001 per minute. All assays were performed at 25 °C, and the absorbance was measured using microplate spectrophotometry. In Fig. 3, the peroxidase activity of the LOE extracted from *S. cerevisiae* modified to overproduce GPX1, GPX2, and GPX3 was measured to be 1.73, 1.99, and 1.10 U/mg, respectively. The peroxidase activity of LOE, isolated from the control *S. cerevisiae*, was 1.09 U/mg, and GPX1, GPX2, and GPX3 overproducers each had 59, 83, and 1% higher activity, respectively, than mock (Fig. 3b). However, the peroxidase activity of the whole protein was measured at similar values in all strains (Fig. 3c). As a result, the LOE activity was high in the order of overproduction of GPX2, GPX1, and GPX3, and all of them were higher than in the control, but the peroxidase activity of the whole protein was similar. These results indicate that the overexpression of a GPX protein stimulated the augmentation of the peroxidase activity of the lysosome fraction.

Melanin decolorization activity of LOE isolated from recombinant *S. cerevisiae*

One hundred parts per million of the melanin solution was mixed with 4 types of LOE isolated from different strains, MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3. The samples were mixed with melanin solution at a concentration of 10% (v/v) and were then reacted at room temperature for 10 days. The melanin content was measured before and after the reaction through the absorbance at 450 nm

Fig. 4 Melanin color reduction by LOE isolated from recombinant *S. cerevisiae*. GC0, GC1, GC2, and GC3 were treated with LOE extracted from MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3, respectively. Control: no treatment. MBTL-GC0 is the mock strain. Data are presented as the means ± SD (*n* = 3). **P < 0.01 and ***P < 0.001 vs. untreated control

with a melanin calibration curve. In Fig. 4, the control was only the melanin solution with no treatment. Other melanin samples were treated with LOE extracted from the recombinant yeasts MBTL-GC1, MBTL-GC2, and MBTL-GC3. These strains were modified to overproduce GPX1, 2, or 3. LOE isolated from MBTL-GC0 (mock strain) was used as a positive control. Regarding the control sample, only 10% of the melanin was naturally decolorized after 10 days, while 53%, 65%, and 49% of the melanin decreased in the GC1, GC2, and GC3 strains, respectively. Compared to a mock strain with a 45% decrease in melanin concentration, the LOE extracted from the GPX1, 2, and 3 strains increased the melanin decolorization activity of 17.8%, 44.4%, and 8.9%, respectively. The reduction of the melanin color for the three recombinant *S. cerevisiae* was related to the peroxidase activity of the LOE. These results confirmed that recombinant yeasts had a higher peroxidase activity than the control group, which led to better melanin degradation. Additionally, the strain that overexpressed GPX2 protein was the most effective strain to reduce the melanin color in *S. cerevisiae.*

Melanin color reduction by LOE in artificial tissue

This experiment verified the whitening effect on the actual skin and the enzyme's corneous tissue penetration potential in the skin through a melanin decolorization assay performed on artificial tissue with the MelanoDerm skin model (MEL-B). The MelanoDerm skin model is a viable reconstituted three-dimensional human skin equivalent containing normal, human-derived epidermal keratinocytes and melanocytes that have been cultured to form a multilayered

structure (Zhang and Michniak-Kohn 2012). The artificial tissues were cultured using the manufacturer's protocol and were treated with LOE isolated from recombinant *S. cerevisiae*. Ten percent of LOE was treated every other day for 16 days, and then, the tissue was homogenized. After homogenization, the melanin content of the mixture containing significantly homogenized tissue was measured through the absorbance at 450 nm. The mock strain's LOE reduced melanin by 5% compared to the control that did not possess anything, and LOE of GPX1, GPX2, and GPX3 overproducers reduced melanin by 26%, 31%, and 18%, respectively. Also, in the positive control treated with 1% of kojic acid, 40% lower amount of melanin was observed compared to the control group (Fig. 5). In the histological analysis, the distribution of the melanin compounds in the tissue was confirmed, and the data was consistent with the trend of the melanin content assay (Fig. 6).

Discussion

One of the major challenges in a skin whitening cosmetic field is to remove or decolorize already produced melanin compounds in the skin. Melanin is a main factor in determining the skin color; therefore, if too many melanin compounds are present in the skin, they make skin unclean and dark and even can cause hyperpigmentation (Mason 1948). Most whitening agents such as kojic acid, ascorbic acid, arbutin, and hydroquinone inhibit melanin synthesis, so they take a long time to show their effects and do not effectively improve pigmentation (Pillaiyar et al. 2017; Pandya and Guevara 2000). To solve these problems, we researched melanin decolorization by lysosomal enzymes that digest
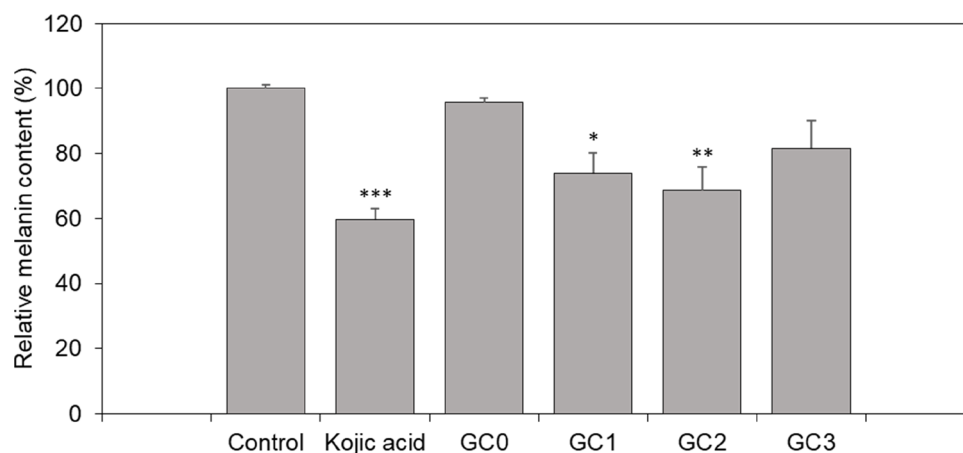

Fig. 5 Melanin content of artificial tissues (MelanoDerm, MatTek Corp, Ashland, MA) after treatment of LOE for 16 days. GC0, GC1, GC2, and GC3 were treated with 10% (v/v) of LOE extracted from MBTL-GC0, MBTL-GC1, MBTL-GC2, and MBTL-GC3, respectively. Control was no treatment, and kojic acid was treated at a concentration of 1% (v/v). GC0 is the mock strain. Data are expressed as a percentage of the untreated control tissues and presented as the means ± SD ($n=4$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. untreated control

Fig. 6 Cross section of stained tissues. The tissue is MelanoDerm artificial skin model, a viable reconstituted three-dimensional human skin equivalent containing normal, human-derived epidermal keratinocytes (NHEK) and melanocytes (NHM) derived from African-American. (**a**) Control not treated. (**b**) Positive control treated with 1% of kojic acid. Others were treated with 10% (v/v) of LOE isolated (**c**) MBTL-GC0, (**d**) MBTL-GC1, (**e**) MBTL-GC2, and (**f**) MBTL-GC3. Tissue was stained by H&E staining, after the reaction. Melanin amount showed decreased by LOE and kojic acid (yellow arrow). MBTL-GC0 is the mock strain
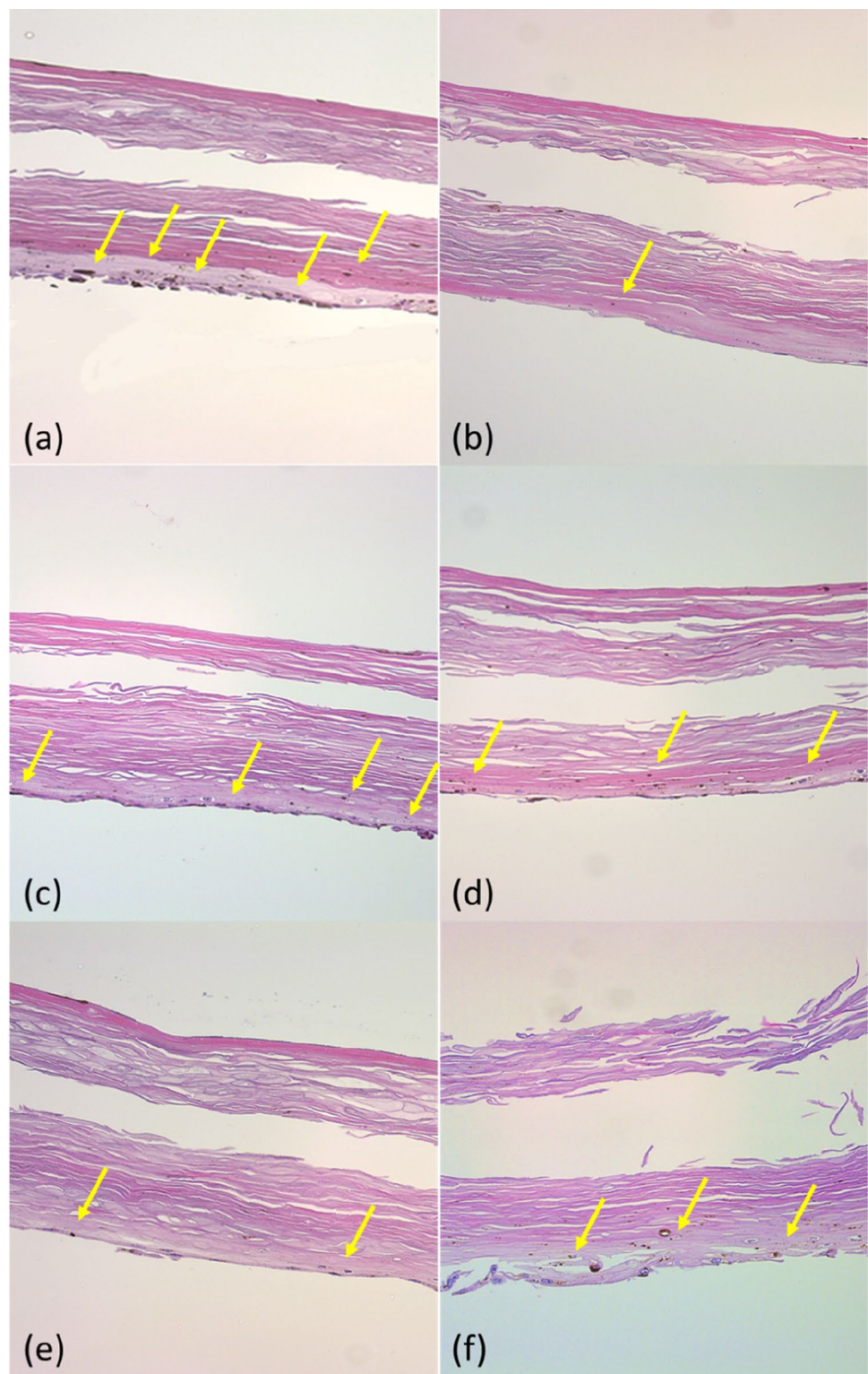
melanosomes in the autophagy system of keratinocytes (Murase et al. 2013).

In the previous research, to separate the lysosome fraction from the cells, LOE was isolated using cell fractionation (Yoon et al. 2015). Then, we found that melanin decolorization of the lysosomal fraction was related to the peroxidase activities of the LOE. GPX enzyme was effective in reducing the melanin color and inhibited the melanin synthesis in the B16F10 melanoma cell (Yoon et al. 2015). LOE is a non-membrane bound microsomal fraction, including

lysosomes and peroxisomes, and the lysosome fraction separated by this method was identified through a lysotracker, fluorescence microscopy, electron microscopy, and lysosomal activity analysis. Additionally, GPX is located at the peroxisome and in the cytoplasm in *S. cerevisiae* and contributes to the antioxidant activity of the cells (Ohdate and Inoue 2012). In this study, to analyze the improvement of the melanin depigmentation activity of LOE by intracellular GPX production, *S. cerevisiae* was modified to overexpress GPX1, GPX2, and GPX3. Then, we analyzed the melanin-decoloring performance and peroxidase activity of the LOE isolated from recombinant yeasts.

In the artificial skin tissue test, the LOE isolated from the GPX overexpressed clones could decolorize melanin compounds in the artificial tissue model, but the LOE of control strain was not effective. The results mean that the GPX plays an important role in the melanin-decolorizing function in the actual skin tissue and support other paper on the use of antioxidants as a treatment for pigmentation caused by oxidative stress (Nahhas et al. 2019).

We used kojic acid as a positive control in the tissue test. Kojic acid is used as a skin-lightening agent commercially. Applied topically, it inhibits tyrosinase and thus prevents the formation of melanin (Burnett et al. 2010). Because of the small molecular size of kojic acid, it seems that the melanin bleaching effect of the positive control group was much better than that of LOE extracted from yeast. In real human skin, the outermost stratum corneum is tightly bound by several binding proteins like a filaggrin (Bos and Meinardi 2000). Because of these binding proteins, large molecules such as proteins are difficult to penetrate through the skin. Therefore, the kojic acid with a size of 142 Da can easily access internal melanocytes and keratinocytes, but enzymes with a size of more than kDa do not (Bos and Meinardi 2000; Sandilands et al. 2009). The skin penetration is one of constant and important problems related to a topical treatment in a cosmetic or medicine field; therefore, many efforts are needed to overcome the limitation. In the overall results, the reduction in the melanin color with LOE of the GPX2-containing strain was the highest in both the melanin compounds bleaching test and the artificial skin tissue test, and the data tendencies of the peroxidase activity, melanin decolorization activity, and artificial tissue tests were identical. This result supports the hypothesis that peroxidase activity is related to melanin-decolorizing activity, and the LOE has potential to function effectively in actual skin by passing through the stratum corneum.

In summary, we suggest that LOE and GPX can be used as a novel skin whitening agent, unlike others that inhibit melanin synthesis. Additionally, further studies will be conducted to analyze melanin degradation intermediates to determine whether the melanin decolorization performed in this paper is related to the degradation of melanin compound.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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