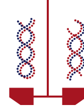
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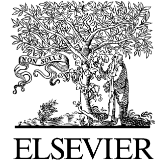
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Inhibitory effects of autolysate of *Leuconostoc mesenteroides* isolated from *kimoto* on melanogenesis

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We investigated the inhibitory effects of the autolysate of *Leuconostoc mesenteroides*, a lactic acid bacterium isolated from sake mash, on melanogenesis in B16F0 murine melanoma cells and a human skin model. The autolysate: induced a decrease in melanin content in B16F0 murine melanoma cells and a 17%, 36%, 41% and 58% decrease in the human skin model by the application of 0.125, 1.25, 6.25, and 12.5 mg/tissue in total; decreased tyrosinase activity to 71%, 46% and 29% of control in B16F0 cells with 0.1, 0.2 and 0.4 mg/ml-medium respectively, but did not inhibit tyrosinase activity under cell-free conditions; decreased amount of tyrosinase in a dose-dependent manner from 74% with 0.1 mg/ml to 33% with 0.4 mg/ml; and decreased amount of tyrosinase mRNA to 80–90% with 0.2–0.4 mg/ml-medium. As the decrease in tyrosinase mRNA levels could not fully account for the reduction in protein, we suggest that the autolysate had post-transcriptional effects rather than transcription inhibition. Our results indicate that the autolysate of *L. mesenteroides* has potential therapeutic use as an effective anti-melanogenic agent.
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[**Key words:** *Leuconostoc mesenteroides*; Melanogenesis; Tyrosinase; B16F0 murine melanoma cell; Human skin model]

Mammalian skin color is determined by the melanogenic activity of melanocytes. Melanocytes are a minor subset of cells located in the skin at the dermal–epidermal interface that specialize in the synthesis and distribution of the pigmented biopolymer, melanin (1,2).

Melanin plays an important role in protecting human skin from UV-induced damage (2). However, overproduction and accumulation of melanin in specific parts of the skin can cause hyperpigmentation disorders such as melasma, freckles, and ephelides. These may become esthetic problems and are a serious concern, particularly in Asia. Thus, a number of lightening agents have been developed for use as cosmetic additives (3–5). The active ingredients in traditional depigmenting products include arbutin, kojic acid, and hydroquinone. Hydroquinone is considered the gold-standard among topical treatments for hyperpigmentation. However, its use is associated with a number of adverse effects, such as cytotoxicity in melanocytes and mutagenesis in mammalian cells (6,7). These data indicate the need to develop safer alternative products to address hyperpigmentation problems.

Melanogenic activity is determined by melanosomes which are membrane-bound granules that form within melanocytes. Melanosomes contain matrix proteins upon which melanin is deposited, and enzymes such as tyrosinase which regulate the biosynthesis of melanin (8). Tyrosinase catalyzes the initial and rate-limiting step in melanogenesis. In this step, the amino acid tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA), which is then oxidized to L-3,4-dihydroxyphenylalanine quinone (L-dopaquinone) (9). Traditional lightening agents are competitive inhibitors of the tyrosinase enzyme system (10,11).

Spontaneous lactic acid bacteria are microorganisms used to attain acidic conditions and prevent the propagation of undesired microbes during the making of traditional seed mash (*kimoto*) when brewing Japanese sake. As many sake brewing workers have good skin, we were inspired to screen lactic acid bacteria isolated from *kimoto* in an attempt to identify novel, natural, and safe tyrosinase inhibitors. Lactic acid bacteria in *kimoto* microflora consist mainly of *Lactobacillus sakei* and *Leuconostoc mesenteroides* (12). Traditionally, organic solvents have been used to extract compounds from microorganisms. However, from a dermatological standpoint, it would be recommended to use the effective substance as an aqueous solution due to the safety against human skin, if the effective substances are soluble not in organic solvent but in water. We found the usefulness of self-digestion (autolysis) to extract and separate the active ingredients from lactic acid bacteria isolated from sake brewing process (13).

In the present study, we demonstrate that the autolysate of *L. mesenteroides* isolated from *kimoto* decreases amount of tyrosinase and melanin in B16F0 melanoma cells and a human skin model, with no cytotoxicity.

MATERIALS AND METHODS

Preparation of lactic acid bacteria LK-103 autolysate Lactic acid bacteria LK-103 were cultured at 30°C for 24 h in MRS broth (Difco Laboratories, Detroit, MI, USA). After cultivation, LK-103 cells were collected by centrifugation, washed twice

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with sterilized water, and re-suspended in sterilized water (1% v/v). Subsequently, cells were autolyzed at 40°C for 72 h. The supernatant obtained by centrifugation was autoclaved and freeze-dried.

Cell culture B16F0 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO_2.

Inhibitory effect on melanogenesis using B16F0 murine melanoma cells B16F0 murine melanoma cells were seeded in a 60 mm dish and cultured with no addition, LK-103 autolysate (0.1, 0.2 or 0.4 mg/ml), or kojic acid (0.028 or 0.14 mg/ml) for 72 h. Cells were trypsinized, counted, and the colors of the cells were evaluated visually.

Determination of the cell viability of B16F0 murine melanoma cells Cell viability was determined by the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (14). Cells were seeded in 96-well plates and cultured for 72 h with no addition, LK-103 autolysate (0.1, 0.2 or 0.4 mg/ml), or kojic acid (0.028 or 0.4 mg/ml). Subsequently, MTT was added to each well, cells were incubated for 3 h, and 10% sodium dodecylsulfate (SDS) was added to dissolve the formazan. The absorbance was measured at 550 nm with a plate reader.

Tyrosinase activity The DOPA oxidase activity of tyrosinase was assessed by a method described in a previous report with some modifications (15). Briefly, B16F0 murine melanoma cells were cultured with DMEM containing 10% FBS and washed twice with ice-cold Dulbecco's phosphate buffered saline (PBS). Cells were lysed with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysates were centrifuged at 15,000 ×*g* for 10 min and the supernatants were removed for the cellular tyrosinase assay. The reaction mixture consisted of 50 μl supernatant, 50 μl freshly prepared substrate solution (0.1% L-DOPA in PBS), and 50 μl sample (0.1, 0.2 or 0.4 mg/ml LK-103 autolysate, 0.028, 0.14 or 0.28 mg/ml kojic acid, or PBS as a control). The reaction mixture was incubated at 37°C for 30 min and tyrosinase activity was monitored by measuring the absorbance at 450 nm.

Determination of the melanin content of a cultured human skin model A human skin model (MelanoDerm; MatTek Corporation, Ashland, MA, USA) was maintained using EPI100LLMM medium at the air/liquid interface according to the manufacturer's instructions. LK-103 autolysate was dissolved in water to give final concentrations of 1, 10, 50 and 100 mg/ml and a 25 μl aliquot was applied directly to the surface of the 0.5-cm^2 tissues on days 0, 3, 7, 11 and 15 (i.e., 0.125, 1.25, 6.25, and 12.5 mg/tissue in total respectively); treatments lasted for 17 d. Water and 10 mg/ml kojic acid were the negative and positive controls, respectively.

After 17 d in culture, tissues were washed three times with PBS, and the cells were solubilized in 2 N NaOH at 80°C for 2.5 h. The solution was centrifuged at 15,000 ×*g* for 20 min and the absorption of the supernatant was determined at 405 nm.

Determination of the cell viability of a cultured human skin model The human skin model (MelanoDerm) was cultured as described above. Cell viability was measured by the MTT assay according to manufacturer's instructions.

DOPA staining of electrophoresed gels (tyrosinase zymography) DOPA staining of electrophoresed gels was performed as previously described (16,17). B16F0 murine melanoma cells treated with no addition, LK-103 autolysate (final concentrations of 0.1, 0.2 or 0.4 mg/ml), and kojic acid (final concentrations of 0.14 mg/ml) for 72 h were solubilized by heating in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Protein content was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), and 30 μg total protein from each cell lysate were resolved by 7.5% SDS−polyacrylamide gel electrophoresis. Gels containing tyrosinase activity were rinsed in 200 ml 0.1 M sodium phosphate buffer (pH 6.8) and equilibrated at room temperature with gentle shaking. After 30 min, the rinse buffer was replaced with fresh buffer. After repeating the rinse procedure, the gels were transferred into 200 ml of staining solution containing the rinse buffer supplemented with 5 mM L-DOPA, and incubated in the dark for 3 h at 37°C. Tyrosinase activity was visualized in the gels as dark melanin-containing bands. The intensities of the bands were determined by ImageJ (National Institutes of Health, Bethesda, MD, USA).

Analysis of amount of tyrosinase by western blotting Western blot analysis of tyrosinase was performed as previously described with some modifications (18). B16F0 murine melanoma cells treated with LK-103 autolysate at final concentrations of 0.1, 0.2 and 0.4 mg/ml for 72 h were solubilized in 0.1 M sodium phosphate buffer (pH 7.2) containing 1% Triton X-100, 0.01% SDS, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Total proteins (20 μg) were resolved by 7.5% SDS−polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) (19). The membrane was blocked with 5% skim milk at room temperature for 1 h and incubated with anti-tyrosinase antibody C-19 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-actin antibody I-19 (1:10,000 dilution; Santa Cruz Biotechnology). After 1 h, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) and the binding antibodies were detected using enhanced chemiluminescence (GE Healthcare) according to manufacturer's instructions. The intensities of the bands were determined using ImageJ.

Total RNA extraction Total RNA was extracted using a RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of the total RNA sample was evaluated by the OD_{260}/OD_{280} ratio.

Reverse transcription Total RNA (1 μg) was extracted from B16F0 murine melanoma cells cultured with no addition or LK-103 autolysate at final concentrations of 0.1, 0.2 or 0.4 mg/ml, and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each cDNA was stored at −20°C.

Analysis of amount of tyrosinase mRNA by real-time PCR Real-time PCR was performed in Applied Biosystems 7500 Fast Real-Time PCR System in a 96-well optical plate. A 1 μl volume of each cDNA was used in 20 μl reaction mixture containing TaqMan® Gene Expression Assays (Applied Biosystems) for tyrosinase (Mm00495817_m1) or beta-actin (Mm01205647_g1), and TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). The thermal cycling conditions were: 20 s at 95°C, and 40 cycles of 3 s at 95°C and 30 s at 60°C. The ΔΔCt method for relative quantitation of gene expression was used to determine amount of tyrosinase mRNA (20).

Statistical analysis Dunnett's method was used for multiple comparisons with a control group.

RESULTS

Effects of LK-103 autolysate on B16F0 murine melanoma cells First, nine strains of *L. sakei*, nine strains of *L. mesenteroides*, and a *Lactobacillus casei* strain, which were the isolates from sake seed mash, were screened by using methanol extracts of the bacterial cells (the final concentration of 0.1 mg/ml in the medium) for anit-melanogenic effects in B16F0 melanoma cells. *L. mesenteroides* LK-103 showed stably the highest activity among the tested strains. 50−80 mg (dry weight) was methanol extraction yield from *L. mesenteroides* LK-103 incubated in 1 L of MRS broth, while the autolysate was found to give the high yields (150 mg). Both extracts appeared to be composed substantially of polysaccharides by the color reaction using phenolic sulfonic acid, and showed equivalent potency on the depigmentation. So in the subsequent experiments, the freeze-dried autolysate was used. Melanogenesis of B16F0 murine melanoma cells cultured with LK-103 autolysate for 72 h was also inhibited (Fig. 1A). The cell viability was decreased slightly by the addition of 0.14 mg/ml kojic acid or 0.4 mg/ml LK-103 autolysate (Fig. 1B), indicating that the effects of LK-103 autolysate on melanogenesis of B16F0 murine melanoma cells occurred without affecting cell proliferation at 0.2 mg/ml.

Effects of LK-103 autolysate on a cultured human skin model To estimate the effect of LK-103 autolysate on natural human skin, we used a three-dimensional cultured human skin model. Fig. 2A shows a macroscopic view of the cultured human skin after 17 d cultivation with or without LK-103 autolysate. The darkening observed in the control was clearly inhibited by treatment with LK-103 autolysate. Under microscopic observation, there appeared to be fewer darkened melanocytes in a dose-dependent manner (Fig. 2B). The melanin content of tissues was decreased to 83%, 64%, 59% and 42% following treatment with 1, 10, 50 and 100 mg/ml LK-103 autolysate, respectively (Fig. 2C). Statistical significances were not found between the experimental and control groups in the cell viability, indicating no cytotoxicity associated with the treatment of LK-103 autolysate (data not shown).

LK-103 autolysate did not directly inhibit tyrosinase activity in vitro To analyze the mechanism by which LK-103 autolysate inhibited melanin synthesis, we examined its effect on tyrosinase activity using crude cell extracts from B16F0 murine melanoma cells. Kojic acid (positive control) clearly showed an inhibitory effect on tyrosinase activity in a dose-dependent manner, which corresponded to the anti-melanogenesis observed in B16F0 murine

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