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Inhibitory effect of ectoine on melanogenesis in B16-F0 and A2058 melanoma cell lines

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ABSTRACT

Skin injuries, congenital lesions, melasma, Addison's disease and many pigment abnormalities prompt us to search for an effective whitening agent. Ideal whitening agent is a natural compound that can inhibit melanogenesis and has no cytotoxic effects. In a previous study, we have developed an optimum method for the production and characterization of ectoine from a halophilic bacterium isolated from a salt environment in Taiwan was identified as *Marinococcus sp.* In the present study, we screened the whitening properties of the biosynthesized ectoine using mouse and human melanoma cell lines, B16-F0 and A2058. Here, we examined the cell viabilities of melanoma cells after ectoine treatment at various concentrations up to 500 μM. Also, we addressed the melanin synthesis of melanoma cells after treatment with ectoine. The inhibitory effects of ectoine on tyrosinase activity were assessed in both mushroom tyrosinase and cellular tyrosinase. Furthermore, we investigated the type of inhibition of mushroom tyrosinase using Lineweaver–Burk enzyme kinetic. The melanogenesis-related gene expression (tyrosinase, TRP1, TRP2 and MITF) and their protein secretion were determined by the assays of quantitative real-time PCR and western blots, respectively. Our results demonstrated that ectoine is a safe and effective whitening agent, inhibited melanin synthesis, reduced both mushroom tyrosinase and cellular tyrosinase, and had various inhibitory effects on the expressions of melanogenesis-related genes and secretion of proteins in mouse and human melanoma cell lines. Thus, we suggest that ectoine can serve as a useful and safe new agent in cosmetic and clinical applications.

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1. Introduction

Nowadays, the production of whitening agents has a great attention due to the important problems resulting from hyperpigmentation of the skin. Many exogenous factors, especially UV radiation, are important causes in skin abnormalities such as melasma, solar lentigines and ephelides [1,2].

Many lightening agents have been reported as melanin inhibitors such as vitamin C, its ascorbyl form is one of these inhibitors [1,3,4]. Not only Vitamin C, but also kojic acid can inhibit tyrosinase activity [1,5,6]. Kojic acid can act as antioxidant and prevent the conversion of the o-quinone to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and dopamine to melanin [5]. In addition, arbutin was reported as an inhibitor of the tyrosinase activity [1,7]. Besides, magnesium ascorbyl phosphate has been shown to protect against skin damage caused by UV-B irradiation [1,8,9]. However, some of the previously reported agents have cytotoxicity and health hazards such as hydroquinone and mercury-containing products. In spite of its remarkable effect against hyperpigmentation, hydroquinone may result in exogenous ochronosis [1,10]. Thus, cosmetic and medical markets are searching for the effective, safer and biosynthesized whitening agent that can reduce melanogenesis without cytotoxicity [11].

Melanin production occurs inside melanosomes, where tyrosine is converted into L-DOPA or dopaquinone by the catalysis of tyrosinase. The formation of eumelanin or pheomelanin is controlled by the presence or absence of cystine [12–15]. In case of cystine absence, dopaquinone gives cyclodopa (leucodopachrome). Redox exchange occurs between leucodopachrome and dopaquinone to form dopachrome and dopa. Tyrosinase-related protein 2 (TRP2)

Abbreviations: DHICA, 5,6-dihydroxyindole-2-carbixylic acid; DMEM, Dulbecco's modified essential medium; D-PBS, Dulbecco's phosphate buffered saline; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; L-DOPA, 3,4-dihydroxy-L-phenylalanine; TRP1, tyrosinase-related protein 1; TRP2, tyrosinase-related protein 2; MITF, microphthalmia-associated transcription factor.

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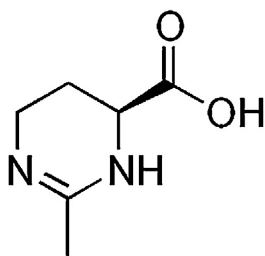

Fig. 1. The structure of ectoine.

catalyses the conversion of dopachrome into 5,6-dihydroxyindole or into 5,6-dihydroxyindole-2-carbixylic acid (DHICA). Tyrosinase-related protein 1 (TRP1) oxidizes DHICA into eumelanin. Otherwise, when cystine is present, dopaquinone is gradually converted into pheomelanin [16,17]. Microphthalmia-associated transcription factor (MITF) is a melanogenesis-related gene. MITF encodes a basic-helix-loop/leucine zipper protein that binds to a symmetrical DNA sequence (E-boxes) (5′-CACGTG-3′) found in the tyrosinase promoter. MITF plays a critical role in the differentiation of various cell types, such as neural crest-derived melanocytes, mast cells, osteoclasts and optic cup-derived retinal pigment epithelium [18]. MITF mutation results in Waardenburg syndrome type IIA, which appears as abnormal pigmentation of the skin and hair [19].

Ectoine is a natural organic compound found in halophilic organisms (Fig. 1). Ectoine helps bacteria against dehydration and osmotic stress [20]. Ectoine-containing formulation can protect skin barrier against the detrimental effect of sodium dodecyl sulfate and can reduce the transepidermal water loss [21]. However, no one has studied in depth the role of ectoine as a whitening agent and melanogenesis inhibitor.

In our previous study, ectoine has been effectively and largely produced in Taiwan using *Marinococcus sp.* isolated from high-sanity environment [22]. Thus, in the present study, we studied the whitening property of ectoine and its effect on two melanoma cell lines; mouse melanoma (B16-F0) and human melanoma (A2058). We determined the cytotoxicity assays, melanin synthesis, mushroom and cellular tyrosinase activities, enzyme kinetic analysis using Lineweaver–Burk plot, expression levels of the melanogenesis-related genes and secretion of the melanogenesis-related proteins after treatment with ectoine up to 500 μM. Our results suggested that ectoine is an effective, non-toxic whitening agent and is an excellent candidate for cosmetic and medical applications.

2. Materials and methods

2.1. Materials

Ectoine was produced and purified according to our previous study [22]. Melanin, L-DOPA, L-tyrosine, Triton X-100, mushroom tyrosinase were purchased from Sigma (St. Louis, MO). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (D-PBS) were obtained from Hyclone (Logan, UT). Arbutin was from Chromadex (Lake Forest, CA). TRIzol reagent and trypan blue stain were purchased from Invitrogen (Carlsbad, CA). RT buffer and Rever Tra Ace were obtained from TOYBO (Shiwa, Japan). SYBR Green Mix was purchased from KAPA BIOSYSTEMS (Boston, MA). 40% acrylamide and TEMEM were purchased from BIO-RAD (Hercules, CA). Prestained protein ladder was purchased from GOGRNE. ECL and antibodies of western blot analysis were obtained from MILLIPORE (Billerica, MA).

2.2. Cell culture

B16-F0 (BCRC Number: 60029) and A2058 (BCRC Number: 60240) were purchased from Bioresource Collection and Research Center (BCRC), Taiwan. B16-F0 and A2058 were grown in culture using DMEM supplemented with 10% FBS. Both cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cell viability assay

Ectoine was used to investigate the toxic injury to cells. B16-F0 or A2058 (1×10^4 cells/well) were plated in 24-well plates in DMEM with 10% FBS for 24 h. The medium of the experimental groups was dropped by varied concentrations of ectoine (0, 10, 50, 100, 200 and 500 μM), followed by incubation for 48 h. After incubation, 0.1 ml of trypsin was added to each well for 3 min, then DMEM containing 10% FBS were added and cells were collected for counting by the Countess™ [23].

2.4. Determination of melanin synthesis

B16-F0 or A2058 cells were cultured at 1×10^4 cells per well in 24-well plates and L-DOPA was added. After 24 h of incubation, the L-DOPA was removed and washed by D-PBS twice. Then the cells were treated with varied concentrations of ectoine (0, 10, 50, 100, 200 and 500 μM) for 48 h. After treatment, the cells were washed by D-PBS twice and dissolved in 100 μl NaOH (1.0 N) for 30 min. OD value was measured using a microplate reader at 492 nm. The melanin content was calculated using an authentic standard of synthetic melanin. [24].

2.5. Inhibition of tyrosinase activity

In addition to cellular tyrosinase (from B16-F0 and A2058), mushroom tyrosinase was used for this assay because of its commercial availability. L-DOPA was used as substrates in this experiment. The activity of tyrosinase was determined spectrophotometrically by monitoring dopachrome formation at 495 nm. In short, 10 mM L-DOPA solution (27 μl) was mixed with the standard solution (150 μl). The mixture was added to various concentrations of ectoine (0, 10, 50, 100, 200 and 500 μM). The tyrosinase solution (200 units, 1 ml) was then added to the mixture for 10 min in a total volume of 200 μl and the absorbance was measured at 495 nm to determine the initial rate of increase in the dopachrome concentration [25].

2.6. Enzyme kinetic analysis

Various concentrations of L-tyrosine (0, 25, 50 and 100 μM) as substrate, 20 μl per well of aqueous solution of mushroom tyrosinase (1000 U/ml), and 34 mM potassium phosphate buffer (pH = 6.6) were added to a 96-well plate in a total volume of 200 μl per well for the assay mixture. The initial rate of dopachrome formation from the reaction mixture was determined as the increase of absorbance at wavelength 495 nm per min by using a microplate reader. The Michaelis constant (K_m) and maximal velocity (V_{max}) of the tyrosinase activity were determined by Lineweaver–Burk plot using various concentrations of L-tyrosine substrate [26].

2.7. RNA isolation and real-time PCR

The total RNA was isolated from B16-F0 and A2058 cells using TRIzol reagent. First-strand cDNA synthesis was carried out using an RT Premix kit according to the manufacturer's instructions. For real-time PCR, primers of tyrosinase, TRP1, TRP2, MITF and

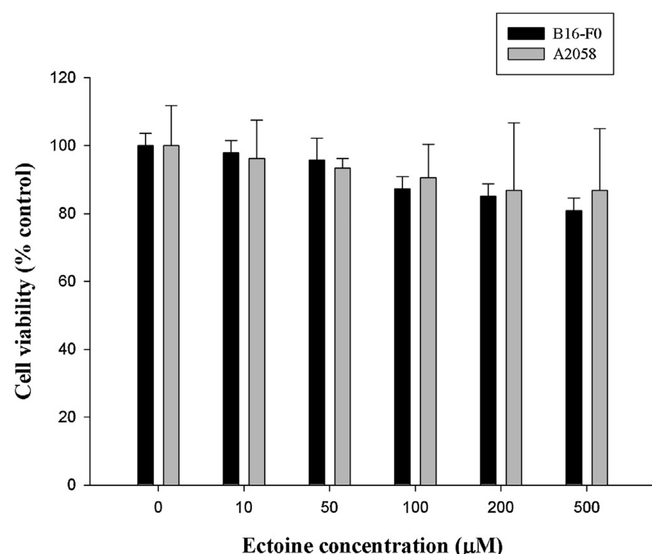

Fig. 2. Cell viabilities were determined by trypan blue after treatment with various concentrations of ectoine. The cells were cultured in 24-well plates and incubated for 3 days. Both cell viabilities of B16-F0 and A2058 were over 80% at 500 μM ectoine.

glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene for normalization) were used. Primer sequences were as follows: tyrosinase, 5′-CAGGTACAGGGATCTGCCAAC-3′ (forward) and 5′-GGTGCATTGGCTTCTGGATAA-3′ (reverse); TRP1, 5′-GCTTTTCTCACATGGCACAG-3′ (forward) and 5′-GGCTCTTGCAACATTTCCTG-3′ (reverse); TRP2, 5′-CGACTCTGATTAGTCGGAACTCA-3′ (forward) and 5′-GGTGGTTGTAGTCATCCAAGC-3′ (reverse); MITF, 5′-CCGTCTCTCACTGGATTGGTG-3′ (forward) and 5′-CGTGAATGTGTGTTCATGCCTGG-3′ (reverse) and GAPDH, 5′-ACCACGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCACCCTGTTGCTGTA-3′ (reverse). A real-time PCR with the SYBR green mix was carried out on an iCycler iQ real-time detection system (Bio-Rad, Hercules, CA). The specificity of the primers was confirmed from a single peak of the melting curve. Each target mRNA level was evaluated from the real-time threshold cycle and compared with the glyceraldehyde 3-phosphate dehydrogenase amount as an internal control.

2.8. Western blot analysis

After the medium had been removed, the cells were washed twice with cold D-PBS. The cells were put on the cold-pillow and then 50 μl Lysis buffer was added. The cells were collected and centrifuged at 14,000 rpm for 15 min. Supernatant was frozen at −80 °C for the following assays. 20 μg of proteins were mixed with 3.3 μl of Dye Reagent and Lysis buffer. The same amounts of proteins from each extract were applied to 10% SDS-PAGE, running gel for 15 min on 80 V subsequently change voltage to 110 V for 2 h and then transferred to a membrane were immersed on 5% BSA/TBST including primary antibody and shocking overnight at 4 °C. Membrane was washed by TBST three times following immersion in the secondary antibody solution. Finally, the enhanced chemiluminescence (ECL) detection reagents were used to develop the signal of the membrane [27].

2.9. Statistical analysis

Each experiment was replicated five times. The control and experimental groups were compared with each other by *t*-test and $p < 0.05$ was considered significant.

3. Results

3.1. Effect of ectoine on cell viability

To measure the degree of cytotoxicity of ectoine, we treated both cell lines B16-F0 and A2058 with different ectoine concentrations.

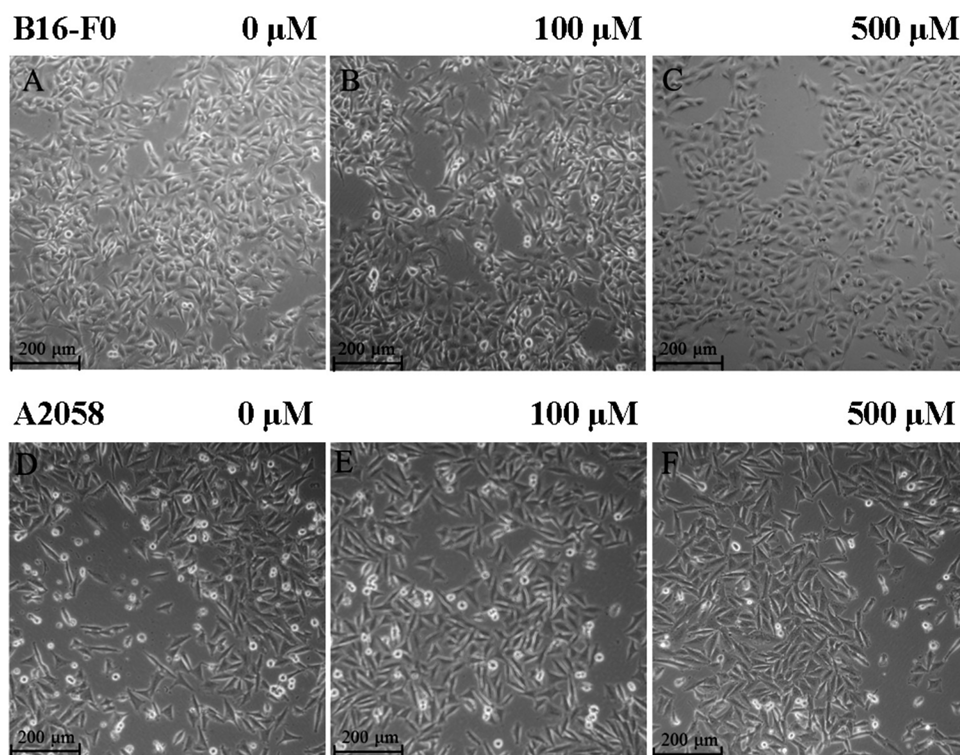

Fig. 3. Representative morphologies of B16-F0 (A) 0 μM, (B) 100 μM, (C) 500 μM and A2058 (D) 0 μM, (E) 100 μM, (F) 500 μM after exposure to ectoine for 3 days.

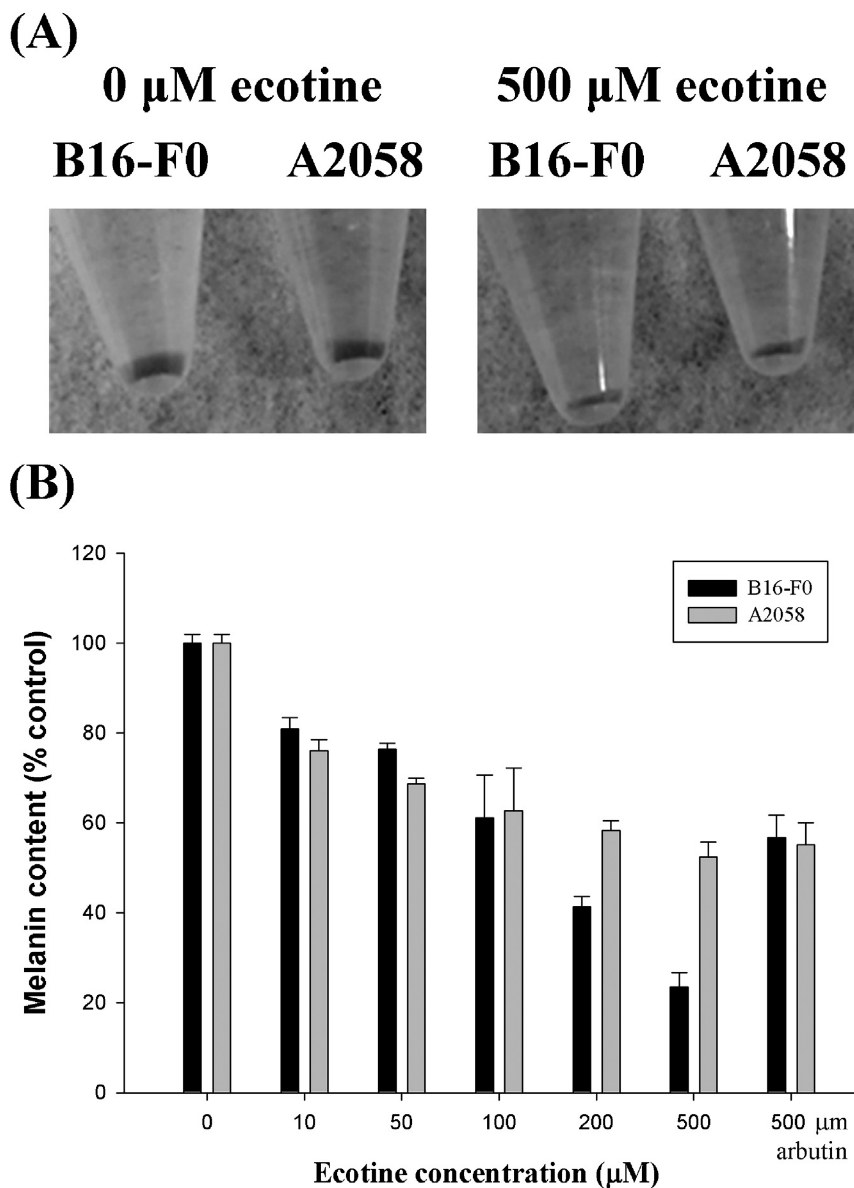

Fig. 4. (A) Showing cell pellets of B16-F0 and A2058 after cells were cultured in the presence of ectoine indicating the inhibitory effect of ectoine on melanin synthesis. (B) Melanin content was measured by spectrophotometer after ectoine treatment at various concentrations. Melanin content in B16-F0 and A2058 decreased when the ectoine concentration increased and reached 23.5% and 52.5% at 500 μM ectoine, respectively. All experimental data with ectoine treatment (10, 50, 100, 200 and 500 μM) were significantly difference with the control (without ectoine treatment, 0 μM).

Cell viabilities were determined using trypan blue exclusion test. As shown in Fig. 2, ectoine has no cytotoxic effect on both cell lines even at high concentration (80.9% and 80.8% viability for B16-F0 and A2058 cell lines at 500 μM ectoine, respectively). Cell morphologies of B16-F0 and A2058 cell lines were not changed after exposure to ectoine for 3 days (Fig. 3).

3.2. Effect of ectoine on melanin synthesis

To determine the effect of ectoine on melanin synthesis, B16-F0 and A2058 cell lines were treated with ectoine at various concentrations and then melanin content was determined by spectrophotometer. As shown in Fig. 4, relative melanin content in B16-F0 was strongly declined reaching 23.5% at 500 μM ectoine (compared with 56.8% for arbutin at the same concentration). Similarly, relative melanin content in A2058 was dropped down to 52.5% at 500 μM ectoine. The IC_{50} of ectoine for the inhibition of melanin contents in B16-F0 and A2058 cells lines were 156.5 μM and 526.2 μM, respectively.

3.3. Effects of ectoine on tyrosinase activities

To address the effects of ectoine on tyrosinase activities, mushroom and cellular tyrosinase (isolated from B16-F0 and A2058 cell lines) were used in vitro. Ectoine caused a significant reduction in the relative mushroom tyrosinase activity reaching to 49.7% at 500 μM ectoine (Fig. 5A). The IC_{50} of ectoine for the inhibition of mushroom tyrosinase activity was 487.16 μM. Similarly, ectoine had aggressively affected on cellular tyrosinase activity reaching the highest decline at 500 μM; 49.8% and 60.2% for B16-F0 and A2058, respectively (Fig. 5B). The IC_{50} of ectoine for the inhibition of cellular tyrosinase activities in B16-F0 and A2058 cells lines were 628.6 μM and 470.8 μM, respectively.

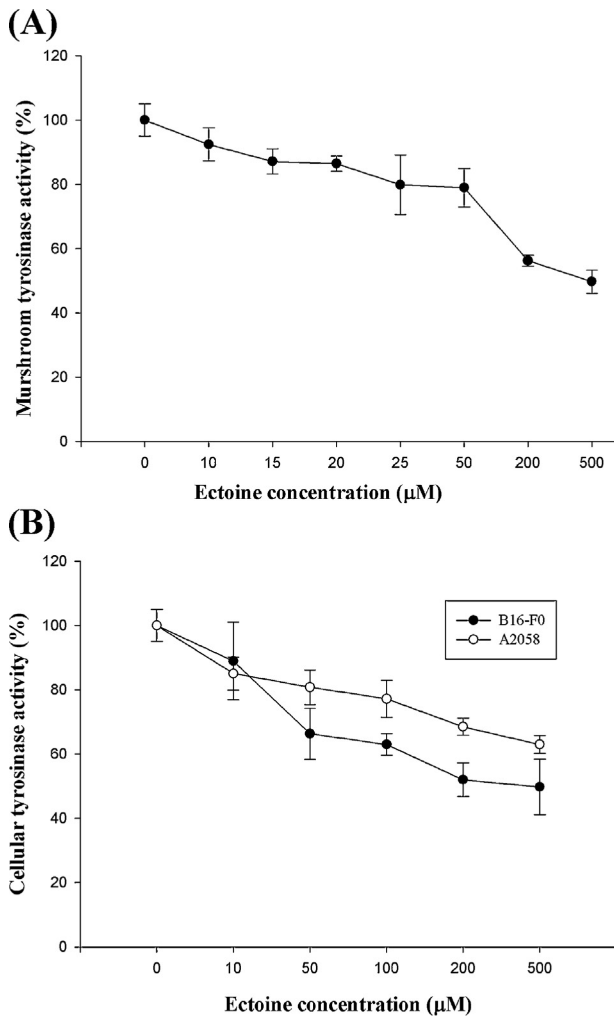

Fig. 5. The mushroom tyrosinase activity was measured after ectoine treatment. Ectoine had caused a significant reduction in the mushroom tyrosinase activity reaching to 49.7% at 500 μM ectoine (A). Similarly, ectoine had aggressively affected on cellular tyrosinase activity reaching the highest decline at 500 μM; 60.2% and 49.8% for B16-F0 and A2058, respectively (B). All experimental data with ectoine treatment (10, 50, 100, 200 and 500 μM) were significantly difference with the control (without ectoine treatment, 0 μM).

3.4. Enzyme kinetics

To investigate the inhibition type of ectoine on mushroom tyrosinase, we used the apparent Michaelis constant (K_m) and maximum velocity (V_{max}) to construct the Lineweaver–Burk plot and to analyze the inhibition type of ectoine on mushroom tyrosinase (Fig. 6). Our result showed that the inhibition type was non-competitive inhibition.

3.5. Effects of ectoine on the expression of melanogenesis-related genes and secretion of melanogenesis-related proteins

To determine the effects of ectoine on the expression of melanogenesis-related genes (tyrosinase, TRP1, TRP2 and MITF), mRNA levels were measured by quantitative real-time PCR. As shown in Fig. 7, the relative gene expressions of tyrosinase, TRP1 and TRP2 in B16-F0 cell line were significantly down-regulated by ectoine reaching the highest drop at 500 μM ectoine (0.24-, 0.20- and 0.12-fold compared to control, 0 μM ectoine, respectively). On the contrary, gene expression of MITF was slightly down-regulated even at 500 μM ectoine (0.74-fold compared to control). In the case of A2058 cell line, we have observed that the gene expressions of tyrosinase and TRP1 were not affected by ectoine even at the highest concentration (1.12- and 1.10-fold compared to control, respectively). In addition, gene expression of TRP2 was slightly declined (0.48-fold at 500 μM ectoine compared to control). However, gene expression of MITF was pointedly decreased (0.19-fold at 500 μM ectoine compared to control). In the meanwhile, the results of secretion of corresponding melanogenesis-related proteins were consistent with the gene expressions (Fig. 8).

4. Discussion

Many whitening agents have been reported and used in cosmetic and medical applications to treat skin and pigment abnormalities. However, some of these agents have cytotoxicity or undesirable side effects such as hydroquinone and mercury. For these reasons, many studies are searching for safe, effective and natural whitening agents.

In a previous study, we had produced ectoine naturally from *Marinococcus sp*. In this study, we screened the whitening properties of ectoine and its effects on both cell lines, B16-F0 and A2058.

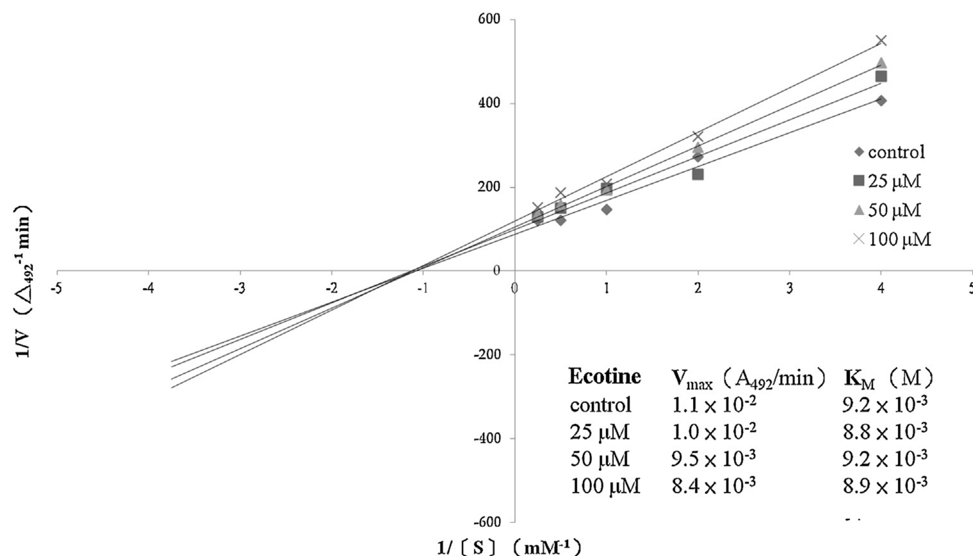

Fig. 6. Lineweaver–Burk plot. Data were obtained as mean values of 1/*V*, of three independent tests with different concentrations of L-tyrosine as a substrate. Inhibitors (ectoine) of the enzyme (mushroom tyrosinase) were 0 (control), 25, 50 and 100 μM. *V* denotes the velocity of the enzyme reaction and *S* for L-tyrosine concentration.

Fig. 7. Quantitative real-time PCR analysis of mRNA expression levels of tyrosinase, TRP1, TRP2 and MITF in both B16-F0 and A2058 cell lines after ectoine treatment.

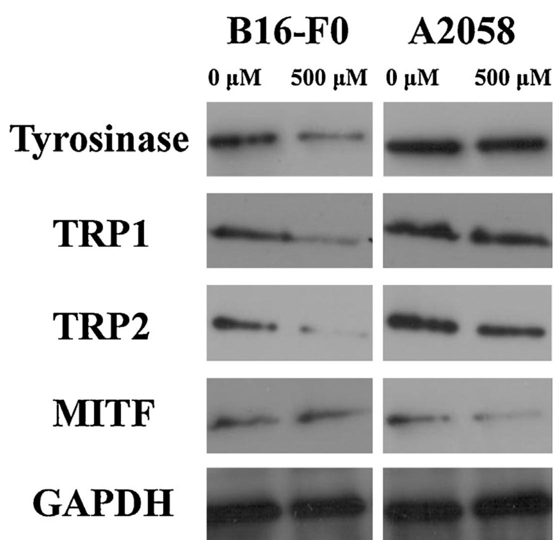

Fig. 8. Western blot analysis of protein secretions of tyrosinase, TRP1, TRP2 and MITF in both B16-F0 and A2058 cell lines after ectoine treatment.

Our results showed that ectoine had no cytotoxicity on both cell lines, B16-F0 and A2058 even at high concentrations (500 µM) (Fig. 2). Our results showed that cell viabilities decrease slightly when the concentration of ectoine increase over 500 µM (approximate 75% for 2 mM of ectoine). Thus, we select safe concentration of ectoine (within 500 µM) that can keep cell viability over 80% for the following assays and studies. Also, ectoine aggressively inhibited the melanin synthesis in both cell lines compared with arbutin (Fig. 4). Tyrosinase is a very important enzyme in melanogenesis pathway. Tyrosinase catalyses the conversion of tyrosine to DOPA and the oxidation of DOPA to dopaquinone [28–31]. In our experiments ectoine deactivated both mushroom tyrosinase and cellular tyrosinase resulting in melanogenesis inhibition (Fig. 5) and the type of inhibition is non-competitive (Fig. 6). Tyrosinase, TRP1 and TRP2 are involved in the melanin production [29,32,33]. Real-time PCR analysis indicated that the expressions of tyrosinase, TRP1 and TRP2 in B16-F0 cells were aggressively dropped down by ectoine treatment. However, only the expression of TRP2 was slightly decreased and the expressions of tyrosinase and TRP1 were not affected by ectoine in A2058 cells.

MITF has an important role in the proliferation and differentiation of melanocytes [34] and is an important transcription factor that can regulate melanogenesis enzymes such as tyrosinase, TRP1, TRP2 [35–37]. Extracellular signal-regulated kinase (ERK) is an important pathway that can control cell differentiation and

proliferation [38,39]. Also, ERK activation can inhibit melanogenesis [40]. MITF phosphorylation through ERK results in MITF degradation [18,41]. In the present study, we demonstrated that the expression of MITF in B16-F0 cells was slightly decreased by ectoine treatment. By contrary, the expression of MITF was strongly down-regulated in A2058 cells by ectoine treatment. Therefore, our results suggested that ectoine is an inducer of ERK pathway and the down-regulation of MITF in both melanoma cell lines had resulted in inhibition of melanin synthesis by deactivation of tyrosinase, TRP1 and TRP2.

5. Conclusions

In summary, we demonstrated the whitening properties of biosynthesized ectoine for the first time and we suggested that ectoine is a putative whitening agent. Moreover, ectoine has been proven by mouse and human melanoma cell lines that ectoine is a safe and potential agent for the cosmetic and clinical application. In the future, more experiments will be necessary to make the mechanism and application of ectoine more completed [42–44].

Conflict of interest

The authors indicate no potential conflicts of interest.

Acknowledgement

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