# OPTIMIZATION AND VALIDATION OF A CELL-BASED TYROSINASE ASSAY FOR SCREENING OF TYROSINASE INHIBITORS

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## ABSTRACT

Tyrosinase is a key enzyme that catalyzes melanogenesis in human skin. It oxidizes tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to dopachrome, which further polymerizes to melanin pigments. Therefore finding an effective tyrosinase inhibitor, either from synthetic or natural sources, is not only useful as skin whitening agents in cosmetic application, but also beneficial in treating melanin-related disorders. The present study reports of the optimized and validated results of a cell-based tyrosinase assay using B16F10 murine melanoma cell line, which produces melanin pigments and has been used extensively in antimelanogenesis studies. The optimization studies involved 3 parameters (1) optimal seeding cell number per well for total protein extraction; (2) optimal dopachrome formation from enzymatic reaction between total protein (tyrosinase source) and L-DOPA (substrate); and (3) optimal incubation period after the addition of substrate. The present study demonstrates that using seeding cell number of $2 \times 10^5$ cells/well, total protein of 40 µg, L-Dopa of 5 mM,and at an incubation period of 1 hour at 37°C provided the optimal response on cultured melanoma cells. Kojic acid, a standard tyrosinase inhibitor, was used as a positive control in the optimized cell-based tyrosinase assay to validate the usefulness of the assay. CONCLUSION: The use of the mentioned protocol is sensitive to determine changes in melanoma cells as the result of tyrosine inhibitors.

***Keywords:*** *Tyrosinase, tyrosinase inhibitors, melanogenesis, melanin, whitening*

## *Introduction*

Melanin production, or melanogenesis, is principally responsible for skin and hair colours, and plays an important defensive role against the harmful effects of ultraviolet radiation of sunlight. However, overproduction and accumulation of melanin result in various skin dermatological disorders including melasma, freckles, age spots, and sites of actinic damage or other hyperpigmentations (1, 2). Melanin is produced normally by melanocytes and, to a greater extent, by melanoma cells. In mammals, melanin is synthesized in the melanosomes of melanocytes that contain tyrosinase, which plays a key role in melanogenesis. Tyrosinase, a copper-containing binuclear enzyme, catalyzes two rate-limiting steps of melanogenesis: that is, the hydroxylation of L-tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to dopaquinone (3). For this reason, tyrosinase is an attractive target in the search for various kinds of whitening agents. Agents that inhibit tyrosinase activity have the potential to be antimelanogenesis agents and thus promote depigmentation, such as hydroquinone and kojic acid (4).

Recently, safe and effective tyrosinase inhibitors have become available which can lead to potential applications in preventing pigmentation disorders and other melanin-related health problems in patients. Tyrosinase inhibitors are also important in cosmetic applications to produce the skin

whitening effects (5). The whitening potential of a compound is commonly measured by inhibiting mushroom tyrosinase in cell-free systems (6, 7). However, it does not adequately represent the whitening effects since it does not account for cellular uptake of the test samples. Several studies have indicated that many plant extracts shows inhibitory activity against mushroom tyrosinase *in vitro* but did not reduce the pigmentation activity in cells (8). Conversely, other compounds tested on cellular tyrosinase in cultured melanocytes did not produce similar results although detected as having tyrosine inhibitory activites(9, 10). Furthermore, it should be noted that some major differences exist between the mushroom tyrosinase and the mammalian tyrosinase, causing many different effects and may result in untoward symptoms in patients (11). Therefore, in order to determine a safe and effective skin whitening agents from natural sources, a reliable, quick and easy method and materials would be needs. In the present paper we described a novel method that led to the development of an optimized cell-based tyrosinase assay using B16F10 murine melanoma cells. B16F10 murine melanoma cells have been widely used to elucidate the regulatory mechanisms of melanogenesis and pigment cell proliferation and thus would serve as a reliable indicator of the effects of the tested material on potential human skin cells.

## *Materials and Methods*

### *Cell culture*

B16F10 murine melanoma cells (CRL-6475) were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 1% (v/v) penicillin/streptomycin (100 units/ml), and incubated at 37°C under 5% $CO_2$ atmosphere. Cells used for experiments were maintained in lower cell passage number (less than 10 passages) to avoid batch-to-batch variations in tyrosinase activity measurement. B16F10 melanoma cells were seeded into 6-well plates for the cell-based tyrosinase assay.

### *Optimization studies*

The cell-based tyrosinase assay described here was designed to incubate the cells with test sample for 72 h at 37°C. The assay was a modification of a previously described method (12, 13) and involved optimizations of some parameters as stated below:

(i) Determination of the optimal seeding cell number per well that yielded sufficient protein containing tyrosinase for the assay;
(ii) Determination of the amount of total protein and concentration of L-DOPA (substrate) required for optimal dopachrome formation; and
(iii) Determination of optimal incubation time for dopachrome formation after the addition of substrate.

For determination of optimal seeding cell number per well, cells were plated in 6-well plates at an increasing seeding cell number ($1 \times 10^5 – 5 \times 10^5$ cells/well) in 3 ml of culture medium without test samples. After an overnight incubation, the medium of each well was replaced with fresh medium. The cells were cultured at 37°C for another 72 h. After 3 days incubation, the cells were harvested and lysed with M-PER mammalian protein extraction reagent (Pierce). The cell lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C and the total protein content was quantified by using a protein assay kit (Bio-Rad). The cell-extracted protein or total protein was used as the source of crude tyrosinase for the tyrosinase assay. Total protein extracted per well increased proportional to the number of cells seeded per well. Seeding cell number that reached confluency on day 5 and gave an adequate amount of total protein (200-400 µg/well) for the tyrosinase assay was determined as the optimal seeding cell number.

After cell lysis on day 5, protein concentrations were adjusted to a range of 20-100 µg/100 µl/well with 0.1 M sodium phosphate buffer (pH 6.8) in a 96-well plate. Then, 100 µl of freshly prepared L-DOPA solution at different concentrations (2.5, 5.0 and 10.0 mM) were added for dopachrome formation. The relationship between total protein and concentration of L-DOPA for dopachrome formation was observed.

The reaction mixture (200 µl/well) consisted cell-extracted protein and L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8) was added into wells of a 96-well plate in triplicates. The plate was incubated at 37°C and absorbance was measured at 475 nm for a time course of up to 4 hours in order to determine the optimal incubation period for measurement of tyrosinase activity. The absorbance of the dopachrome formation increases over incubation time. The changes in absorbance values were monitored every 10 min for the first two hours and every 30 min for the subsequent hours up to a total incubation period of 4 hours.

Kojic acid, a known tyrosinase inhibitor, was used as a positive standard to confirm the efficacy of the optimized cell-based tyrosinase assay. Using the optimized protocol, after 3 days incubation of kojic acid at increasing concentrations (100, 250 and 500 µg/ml) in B16F10 melanoma cells, the tyrosinase inhibitory activity was measured. The values obtained were compared to the data of inhibitory activity of kojic acid reported in other papers. Values are expressed as mean ± standard deviation (SD) of at least 3 independent determinations.

## *Results and Discussion*

A radio-enzymatic method for the measurement of tyrosinase activity was first introduced by Pomerantz (14) and has been extensively employed in studies of melanin synthesis, using tissue homogenates or cultured melanoma cells as substrates. In this study, the tyrosinase assay was applied to a cell culture system using B16F10 melanoma cells for antimelanogenesis study.

According to our optimization results, the amount of total protein extracted from the cells increased proportionally to the initial seeding number of cells per well up to a stage where the cells wver beyond confluent (data not shown). We have selected $2 \times 10^5$ cells/well as the optimal seeding cell number because the cells reached confluency after the incubation periods and produced sufficient protein content for the cell-based tyrosinase assay (triplicates). Dopachrome is formed from the enzymatic reaction between the total protein (tyrosinase source) and L-DOPA (substrate). The amount of dopachrome produced depends on the amount of total protein and the concentration of L-DOPA used in the enzymatic reaction. Figure 1 shows that increasing amount of total protein (20-100 µg) reacts maximally with 5 mM of L-DOPA and gives an increasing absorbance values at 475 nm. It was observed that 5 mM of L-DOPA produced better readings of absorbance values compared to 2.5 mM of L-DOPA. More cells are needed for the assay if higher amount of total protein is chosen for the enzymatic reaction. Therefore, based on the optimal seeding cell number that we have selected and the amount of total protein that can be extracted from the cells, the minimum acceptable dopachrome formation was at 40 µg of protein and 5 mM of L-DOPA. These levels of protein and L-DOPA were adopted for optimal dopachrome formation which produced acceptable absorbance value at 475 nm. A time course study of cellular tyrosinase activity in B16F10 cells up to four hours demonstrated that the dopachrome formation inces overduring incun time at 37°C resulting in an increasing absorbance value at 475 nm (Figure 2). We have adopted one hour as the optimum incubation period for end-point measurement of tyrosinase activity due to the rate of cellular tyrosinase activity was rapid and linear in the first hour and produced acceptable absorbance value at 475 nm.

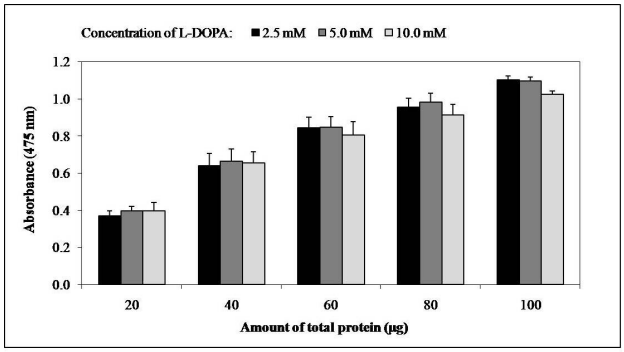


*Figure 1: The relationship between the total protein and the concentration of L-DOPA for dopachrome formation in cell-based tyrosinase assay. Each value represents the mean ± SD (n=3).*

To verify the usefulness of the optimized assay, kojic acid (a standard tyrosinase inhibitor) was tested as a positive control. Kojic acid at concentrations of 100, 250 and 500 µg/ml showed dose-dependant cellular tyrosinase inhibitory activity of 15.44%, 31.23% and 41.37% in B16F10 cells respectively. The inhibitory activity of kojic acid measured by this optimized assay is comparable to the tyrosinase inhibitory activity of kojic acid reported by Im *et al.* (15) on the same cell line, B16F10 melanoma cells. This finding indicates that our optimized cell-based tyrosinase assay, is useful and valid as compared to other reported studies, in which kojic acid was used as a positive control.

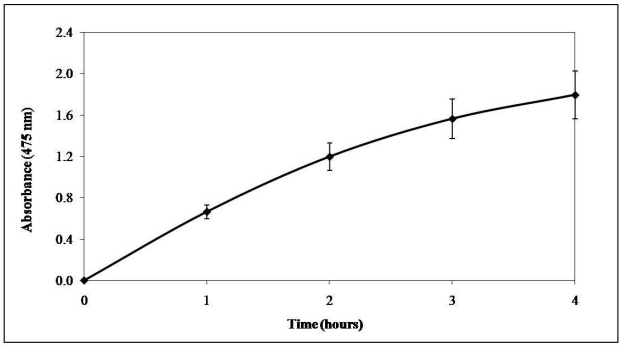


*Figure 2: Time course study of cellular tyrosinase activity using 40 µg of protein and 5 mM of L-DOPA. Each value represents the mean ± SD (n=3).*

## Conclusion

The development of an optimized and standardized cell-based tyrosinase assay using B16F10 melanoma cells may provide useful information to researchers who are using the same cell line for tyrosinase assay. The optimized cell-based tyrosinase assay is highly reproducible, sensitive and offers a relevant screening tool to search for more effective tyrosinase inhibitors from natural sources such as plant extracts.

## Acknowledgement

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## *Discussion with Reviewers*

**Reviewer**: The authors claimed that they have developed an optimized assay for tyrosinase. However, a comparison between their final results and those reported in reference (12, 13) shows little difference.

**Authors**: Optimization of cell-based assay is an important preliminary step to determine optimized condition of the assay for providing relavant results in a laboratory. Current assay was developed and optimized specifically for tyrosinase derived from B16F10 melanoma cells (a subline of B16 melanoma that is highly metastatic). The slight difference in optimization results of our study compared to other studies (Reference 12 and 13) is reasonable as this could be due to the use of different cell line as source of tyrosinase enzyme (reference 12 – use HEMn cells; reference 13 – use B16 melanoma cells) and different laboratory settings.

**Reviewer**: In the paper, the authors reported the 'optimized conditions' as total protein – 40 µg, L-Dopa – 5 mM; incubation period – 1 hour.

Authors: Yes. Our optimized key assay parameters are: total protein – 40 µg, L-Dopa – 5 mM; incubation period – 1 hour.

**Reviewer**: In reference 12, the conditions stated are: protein --40µg, L-Dopa 2.5mM, incubation period -1 hour.

There is essentially no difference between data reported by the authors and reference 12, the reason is, of course, that authors of reference 12 had already 'optimized' the method.

**Authors**: In response to reviewer's comments 3 & 4, the slight difference between the concentration of substrate (L-Dopa) used in our study (5.0mM) and those reported in reference 12 (2.5mM) is reasonable due to the use of different cell line (source of tyrosinase enzyme) and different laboratory settings. At 40µg of total protein, we observed that 5.0mM of L-Dopa reacts maximally and produces better readings of absorbance values compared to 2.5mM of L-Dopa.

**Reviewer**: The authors claimed that the optimum L-Dopa concentration was 5 mM, but from the data presented in the figure provided, statistically there is no difference between the readings of 5 mM and 2.5 mM. In fact, for almost all protein concentrationa, there is no difference in reading whether L-Dopa concentration is 2.5, 5 or 10mM, and the reason is obvious, all this concentrations are above the $K_m$ of the substrate, and when one measures rate of enzyme catalyzed reaction at substrate concentration >> $K_m$ (saturation), varying the substrate concentration will not alter the rate of reaction.

**Authors**: In our study, the amount of tyrosinase enzyme present in the cell-extracted protein or the activity of the enzyme itself is the limiting factor, and not the amount of substrate (L-Dopa) available. This means that the concentration of substrate must be high enough to ensure that the enzyme is acting at $V_{max}$. In practice, it is usual to use a concentration of substrate higher than the $K_m$ in order to determine the activity of an enzyme in sample. It was preferable to use 5.0mM of L-Dopa as it reacts maximally at 40µg of total protein (source of tyrosinase enzyme) and produces better readings of absorbance values compared to 2.5mM of L-Dopa.

**Reviewer**: I must say that what the authors reported are essentially preliminary work before the study of any action of enzyme –ie, setting the enzyme activity determination conditions, and works of this nature are not acceptable for publication. It is just an essential step in study of any enzymatic reaction.

**Authors**: Preliminary optimization of cell-based tyrosinase assay is essential before the study of tyrosinase inhibitory activity for screening of tyrosinase inhibitors. We verified the usefulness of the optimized assay by testing with a positive control – kojic acid (a standard tyrosinase inhibitor). We reported the development of an optimized and standardized cell-based tyrosinase assay using B16F10 melanoma cells which may provide useful information to researchers who are using the same cell line for tyrosinase assay.