RAPID SCREENING OF TYROSINASE INHIBITORS FROM CASHEW (ANACARDIUM OCCIDENTALE) NUT SHELL LIQUID (CNSL) EXTRACT

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Tyrosinase can be inhibited by analogues of its substrate, tyrosine. Cashew nut shell liquid (CNSL) extract from cashew (Anacardium occidentale) contains several natural phenols that can serve as analogue of tyrosine. This study tested the tyrosinase inhibitory activity of CNSL against mushroom tyrosinase and compared it with two other tyrosinase inhibitors, benzoic acid and cyanide. In addition to this, an assay downscaled to microtiter format for measuring spectrophotometric tyrosinase activity assay was also optimized. This optimized method was found to have comparable results with the standard method and it is more advantageous in terms of cost and length of time of analysis. The data showed that CNSL can significantly inhibit tyrosinase activity to a concentration as low as 0.005 mg/mL. The effect of zinc ions on the tyrosinase activity was also studied in the two methods.

INTRODUCTION

Tyrosinase, also known as polyphenol oxidase, is a bifunctional, copper-containing oxidase having both catecholase and cresolase activity (Malmström & Rydén 1968). It is one of the most important key enzymes in the insect molting process (Andersen 1979); by manipulating this molting process using these enzymes, an alternative insect control agent can be discovered. It is responsible for the browning reaction in plants, which results in loss of nutritional value and deterioration of quality of plant-derived foods and beverages (Friedman 1996) and in the synthesis of melanin pigment by melanocytes in mammals (Chen et al 1995).

It also serves as marker of melanocyte differentiation and commonly expressed by malignant melanoma (Jungbluth et al 2000). Because of these, tyrosinase inhibitors have become increasingly important for cosmetic and medicinal products in relation to hyper-pigmentation. Tyrosinase inhibitors may also control production of melanin since tyrosinase is involved in the process of melanin biosynthesis. Thus melanin synthesis inhibitors are used topically for treatment of localized hyper-pigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state and melanoma pregnancy (Joung Ha 2001).

Tyrosinase inhibitors have been obtained from different organisms such as the mushroom Agaricus hortensis (Madhosingh 1974), Bolivian medicinal plants like Buddleia coriacea, Gnaphalium cheiranthifolium and Scheelea princeps (Kubo et al 1995), and fruits of Anacardium occidentale (Kubo et al 1994) and Artocarpus incisus (Shimizu et al 2000).

It is possible that CNSL contains tyrosinase inhibitors since they are present in cashew fruits (Kubo et al 1994), and CNSL has been found to contain natural phenols (Tyman & Morris 1967) that can serve as analogue of tyrosine and inhibit tyrosinase activity (Lejszak et al 1985). This study aims to test the inhibitory activity of CNSL against tyrosinase. In relation to this, the study aims to develop and optimize a microcolorimetric-based assay method for rapid screening of tyrosinase inhibitors.

MATERIALS & METHODS

Cashew nuts were collected from Palawan. Fifty grams of cashew shells were chopped into small pieces and extracted with 200 mL hexane using the Soxhlet apparatus. Extraction was only stopped when the hexane dripping down the flask became clear. Distillation of the collected solution was done by heating using a
mantle until the liquid was boiling mildly. Distillation was stopped when only about 20 mL of the residue was left. The residue was then placed in a beaker and subjected to a steam bath until all the hexane evaporated.

Heating was stopped when the hexane dripping down the flask became clear. The solvent was removed in vacuo using a rotary evaporator. The CNSL obtained was stored in an amber bottle at room temperature (28°C) and shielded from light by storing it in a locker.

Spectrophotometric tyrosinase assay
The tyrosine inhibitory activity was assayed in in vitro conditions following the procedure of Masamoto et al (1980) with modification. Solutions of 1 mg/mL tyrosinase (Sigma Chemical), 0.4 mM L-dihydroxyphenylalanine (DOPA), 0.8 M cyanide, 0.8 M benzoic acid and 0.3 mg/mL and 0.5 mg/mL CNSL were prepared. With 1 mL of the L-DOPA solution, 10 µL of the inhibitor (cyanide, benzoic acid, CNSL) or 10 µL of water (20 µL in the case of the control samples) and 10 µL mushroom tyrosinase (added last) were mixed in a test tube. Phosphate buffer saline (PBS), pH 6.8, was added to the solution for a total volume of 2 mL. The solution was placed in a cuvette, and the change in absorbance at 475 nm was routinely measured every minute for 15 min using a UV-VIS spectrophotometer. The blank used was the sample without the enzyme solution. Three replicates of each sample were subjected to this assay.

Microcolorimetric-based tyrosinase assay
Solutions of 0.1 mg/mL tyrosinase, 0.4 mM L-DOPA, 0.08 M cyanide, 0.08 benzoic acid, 0.0025 mg/mL, 0.005 mg/mL, 0.015 mg/mL and 0.025 mg/mL CNSL-dimethylsulfoxide (DMSO) were prepared. The wells of the microplate were loaded with 10 µL of the inhibitor (cyanide, benzoic acid or CNSL), 10 µL of water (20 µL in the case of the control samples), and 10 µL tyrosinase. A multichannel pipette was used in adding simultaneously 100 µL of the L-DOPA solutions. PBS (pH 6.8) was added to the solution for a total volume of 200 µL. The reader was set for dual wavelength λ₁-λ₂ at 490 nm-650 nm. The change in absorbance at 490 nm was routinely measured every minute for 15 min using a microplate reader (BioRad Model 3550-UV). The blank used was a control sample without the enzyme solution.

Effect of zinc
Zinc ions form zinc nitrate with concentrations of 0.1 M and 0.01 M were prepared. The effect of the metal in the assay was assessed by replacing the 10 µL distilled water in the spectrophotometric tyrosine assay with 10 µL of 0.1 M Zn ions. The same thing was done with the microcolorimetric-based tyrosinase assay where 10 µL of 0.01 M Zn was used instead of 10 µL distilled water.

RESULTS & DISCUSSION

Solvent extraction
The CNSL extract was a brown viscous liquid that was immiscible with polar solvents like water and ethanol. The amount of CNSL extracted from the cashew shells ranged from 11% to 13% (weight by weight). Its specific gravity was 1.1951 g/mL.

Tyrosinase assay
From the preliminary spectrophotometric tyrosine assay, the absorbance at 475 nm progressed linearly up to 6 min ,then leveled off at 15 min. The results indicate that the best time to measure absorbance is from the 1st to 3rd min. It can be observed (Figure 1) that the absorbance of the samples containing 0.3 mg/mL and 0.5 mg/mL CNSL was relatively high during the beginning of the reaction (1st to 3rd min). This can be attributed to the formation of an insoluble precipitate due to the non-polar CNSL. Figure 1 also shows the effectiveness of CNSL in comparison with cyanide and benzoic acid in inhibiting tyrosinase activity. The lower absorbance in the presence of CNSL and the known inhibitors indicates lower concentrations of dopachrome resulting from the oxidation of L-DOPA by the enzyme. Although both cyanide and benzoic acid act as inhibitors, their modes of inhibition are different owing to the presence of two binding sites in the tyrosinase – catechol and oxygen. Cyanide is a non-competitive inhibitor because it competes with oxygen and does not compete with catechol, while benzoic acid is a competitive inhibitor since it competes with catechol (Duckworth & Coleman 1970).

Tyman & Morris (1967) described the composition of cashew nut shell liquid as anacardic acid (71.7%), cardol (18.7%), cardanol (4.7%), novel phenol (2.7%), and two minor ingredients (2.2%). These are natural phenols that may be able to serve as analogues of tyrosine, the enzyme substrate. There has been evidence in the past that suggests that this inactivation is due to the formation of a compound between the enzyme protein and the oxidation product of the analogue (Lejszak et al 1987). Several analogues of tyrosine and L-DOPA have been found to inhibit tyrosinase competitively (Lejszak et al 1987). It is possible that the mode of inhibition of CNSL is competitive. The phenolic compounds of CNSL competitively inhibited tyrosinase the same way benzoic acid competes with catechol for binding sites (Duckworth & Coleman 1970). Another mode of inhibiting tyrosinase is by formation of copper complex but this is not possible since phenolic compounds do not form any complex with copper.

Due to the nonpolar nature of the CNSL extract, DMSO was used to dilute the CNSL extract in the microcolorimetric based method. Because of the composition and structure of DMSO, it can serve as a bridge to carry the CNSL to the enzyme. However, traces of white precipitate were observed when CNSL-DMSO
solution was mixed with polar solutions when the concentration of the CNSL exceeded 0.005 mg/mL. Because of this, the microplate reader was set for dual wavelength at 490 nm-650 nm (\( \lambda_1-\lambda_2 \)) to minimize the absorption due to this precipitate.

The absorbance at 490 nm significantly decreased when the enzyme was in the presence of CNSL with concentrations of 0.005 mg/mL and higher (Figure 2). The effectivity of the extract increased as it became more concentrated. The sample containing 0.025 mg/mL exhibited the most inhibitory effect among all the samples containing CNSL. The CNSL with concentration of 0.005 mg/mL was comparable with that of benzoic acid and those with higher concentrations were found to be more effective inhibitors than benzoic acid but less effective than cyanide.

**Effect of zinc**

Results of the tyrosinase assay (Figure 3) show the potency of zinc ions in catalyzing tyrosinase activity. Addition of zinc ions increased tyrosinase activity. Zinc ions have been found to generate melanochrome formation (Vachtenheim et al 1985). It is interesting to note that a high concentration of zinc ions has been found in subcellular particles in which melanogenesis takes place (Seiji et al 1963).

**Comparison of the two methods**

The results using the standard method and the microcolorimetric method developed were similar. The CNSL was found to exhibit tyrosinase activity in both methods. The formulation of a microcolorimetric-based assay is significant for future studies. Since the microwells in this assay are numerous, the microcolorimetric method provides a fast and efficient way to test tyrosinase activity in large sample sizes. The small quantities of the samples that are required reduce the cost of the reagents used. Because of the nature of the equipment used (Microplate reader), it also opens the options of applying robotics to automate the whole assay, providing an even faster way to test for tyrosinase activity.

**SUMMARY & CONCLUSION**

Tyrosinase inhibitors from the cashew nut shell liquid were successfully detected using the standard method. The tyrosinase inhibitors from CNSL showed comparable activity as that of known tyrosinase inhibitors, cyanide and benzoic acid.

In addition, a rapid, high-throughput, cost-effective microcolorimetric-based assay for tyrosinase inhibitors was successfully optimized. With this method, tyrosinase inhibitors from cashew nut shell oil can be successfully detected using lesser amounts of samples and needing only a shorter time for analysis.
Tyrosinase activity using the microcolorimetric based method developed in the presence of CNSL, cyanide and benzoic acid

Tyrosinase activity using the standard method in the presence of CNSL, cyanide, benzoic acid and zinc ions

**LITERATURE CITED**


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**Figure 4.** Tyrosinase activity using the microcolorimetric-based method developed in the presence of CNSL, cyanide, benzoic acid & zinc ions