Journal of Bioscience and Bioengineering
VOL. 121 No. 1, 13–20, 2016

www.elsevier.com/locate/jbiosc

Inhibitory effect of apocarotenoids on the activity of tyrosinase: Multi-spectroscopic and docking studies

Amrita Anantharaman,[1] Hridya Hemachandran,[1] Rajendra Rao Priya,[1] Mohan Sankari,[1] Mohan Gopalakrishnan,[2] Nallasamy Palanisami,[2] and Ramamoorthy Siva[1,*]

School of Bio Sciences and Technology, VIT University, Vellore 632014, Tamil Nadu, India[1] and School of Advanced Sciences, VIT University, Vellore 632014, Tamil Nadu, India[2]

Received 6 January 2015; accepted 12 May 2015
Available online 14 July 2015

In this present study, the inhibitory mechanism of three selected apocarotenoids (bixin, norbixin and crocin) on the diphenolase activity of tyrosinase has been investigated. The preliminary screening results indicated that apocarotenoids inhibited tyrosinase activity in a dose-dependent manner. Kinetic analysis revealed that apocarotenoids reversibly inhibited tyrosinase activity. Analysis of fluorescence spectra showed that apocarotenoids quenched the intrinsic fluorescence intensity of the tyrosinase. Further, molecular docking results implied that apocarotenoids were allosterically bound to tyrosinase through hydrophobic interactions. The results of the *in vitro* studies suggested that higher concentrations of bixin and norbixin inhibited tyrosinase activity in B16F0 melanoma cells. Our results suggested that apocarotenoids could form the basis for the design of novel tyrosinase inhibitors.

[**Key words:** Apocarotenoid; Norbixin; Bixin; Crocin; Tyrosinase; Food colorant]

Melanogenesis is a highly complex physiological process that results in the formation of melanin pigment (1). The high molecular weight brown pigment melanin has the potential to absorb harmful free radicals generated through ultraviolet irradiation, which in turn cause DNA damage (2). Tyrosinase, a rate limiting multifunctional copper-containing metalloenzyme, plays an essential role in melanin biosynthesis. It catalyzes both monophenolase and diphenolase activities in the melanogenesis process (3–6). On the other hand, the over expression of tyrosinase in human beings leads to various hyper-pigmentation diseases such as freckles, melasma, and age spots (7). Moreover, tyrosinase plays a key role in various biochemical processes of insects such as sclerotization of the cuticle, defensive encapsulation, melanization of foreign organisms, and wound healing (8). Therefore, tyrosinase inhibitors can be used as insecticides in the agricultural field (9). Tyrosinase causes unfavorable enzymatic browning of food products, which results in the loss of organoleptic properties and nutritional quality (5,6). It also reduces the flavor and color of foods, as well as their commercial values (10,11). To overcome these above-mentioned problems, a large number of antityrosinase agents have been derived from natural and synthetic sources (12). As a result, there is a growing need for safe and high-potential tyrosinase inhibitors that can be used in various applications such as in the pharmaceutical, agricultural, food and cosmetic industries (5,13).

Apocarotenoids are isoprenoid compounds formed through oxidative cleavage of carotenoids. More than 100 naturally occurring apocarotenoids with diverse structural and functional properties have been reported. In our daily lives, these apocarotenoids are being used as aroma compounds and as natural pigments (14). The traditionally used orange-yellow pigment bixin, obtained from *Bixa orellana* seed (annatto) is ranked as the second food colorant in the world (15–17). As reported at Joint FAO/WHO Expert Committee on Food Additive (JECFA) (18), the acceptable daily intake (ADI) of annatto extract (containing low concentrations of bixin and norbixin) is 0–0.065 mg/kg body weight . The saponified product of bixin is water-soluble norbixin, which is used as a cheese-coloring agent (19). The compound *cis*-bixin has the ability to interact with human α_1-acid glycoprotein and prevents lipid peroxidation (20). Bixin and norbixin possess anti-carcinogenic potential and also enhance insulin-dependent glucose uptake into differentiated T3-L1 adipocytes (21,22). Zhang and Zhong (19) reported that norbixin interacts with dairy proteins, especially with casein and whey, without affecting the native structure of the proteins. Crocin, a yellow-colored pigment from the saffron stigma, possesses hydrophilic properties. It has been used in various special cuisines, especially for its color, odor, and taste. Apart from its food colorant properties, it is used as an anti-tumor, anti-depressant, cardio-protective, antitussives, genoprotective, and neuro-protective agent (23). Unfortunately, no reports are available on the ADI of crocin or saffron stigma, but many researchers have recognized through in vivo and clinical studies that this natural colorant possesses various pharmacological activities. In rats, it was observed that 1% crocin dye induces reversible liver pigmentation (24). However, in healthy volunteers, no major adverse effect was seen after the consumption of 200 and 400 mg of saffron tablets (25). Asai et al. (26) reported that crocin, when orally administered, was hydrolyzed to crocetin before or

* Corresponding author. Tel.: +91 9443448905; fax: +91 416 2243092.
E-mail address: rsiva77in@rediffmail.com (R. Siva).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.05.007

during gastrointestinal absorption. This crocetin has the ability to affect nucleic acid and protein synthesis in malignant cells, which leads to the finding of an interaction between crocetin and calf-thymus DNA (27).

In our present study, we investigated the mechanism of tyrosinase inhibition by three major apocarotenoids namely bixin, norbixin and crocin through cell-free system using mushroom tyrosinase, kinetic analysis, circular dichroism (CD) technique, intrinsic fluorescence quenching and molecular docking studies. In addition, the effects of apocarotenoids on cellular tyrosinase activity and melanin synthesis were studied through *in vitro* studies using the B16F0 melanoma cell line. In our day-to-day life, these food colorants are consumed without exploring the in-depth mechanism occurring in our human system. Therefore, we carried out this work to gain knowledge on the interaction mechanism of apocarotenoids with tyrosinase, which can be utilized for various industrial applications. Ultimately, these food colorants can be used as a template for the discovery of numerous novel tyrosinase inhibitors.

MATERIALS AND METHODS

Chemicals and reagents Tyrosinase (EC 1.14.18.1) and crocin were purchased from Sigma (St. Louis, MO, USA). L-3,4-Dihydroxyphenylalanine (L-DOPA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Triton X-100 and dimethyl sulfoxide (DMSO) were obtained from Himedia, India. All other chemicals were of highest-purity analytical grade.

Preparation of bixin and norbixin Bixin was isolated from the seeds of *B. orellana* and norbixin was obtained through the saponification of bixin as per the protocol of Kovary et al. (21). Further, the purity of the obtained bixin was confirmed through ultraviolet visible spectrophotometry, Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) spectroscopy. High performance liquid chromatography (HPLC) analysis of bixin was carried out as per the protocol of Mahendranath et al. (28). Similarly, the isolation and characterization of norbixin were performed as already described in our previous report (29).

Cell -free diphenolase enzyme activity The inhibition of tyrosinase activity by apocarotenoids (bixin, norbixin and crocin) were analyzed spectrophotometrically according to the method of Chan et al. (30) with minor modifications. Bixin was dissolved in 0.1 M phosphate buffer (pH 6.8) containing 0.1% DMSO. Norbixin and crocin was solubilized in 0.1M phosphate buffer (pH 6.8). Briefly, the assay mixture containing 50 µl of tyrosinase (700 U/ml), 50 µl of different concentrations of apocarotenoids (5, 10, 25 and 50 µg/ml), 100 µl of 0.1 M phosphate buffer (pH 6.8) was added into each well of a 96-well plate and then this assay mixture was incubated at room temperature for 10 min. After incubation, 100 µl of 12 mM L-DOPA was added into each well and then incubated for 20 min at room temperature. The optical densities of the samples in each well were determined at 475 nm by using Biotek ELX800 absorbance microplate reader. Kojic acid was used as the positive control.

The percentage inhibition of tyrosinase activity was calculated as follows (3):

$$(\%)\ \text{Inhibition} = [1 - (B/A)] \times 100 \quad (1)$$

where A is the absorbance of reaction mixture at 475 nm containing test sample and tyrosinase; B is the absorbance at 475 nm without the test sample.

Kinetic analysis Different concentrations of L-DOPA (2–10 mM) were incubated with a constant concentration of tyrosinase (700 U/ml) and then three different concentrations of apocarotenoids (10, 25 and 50 µg/ml) were added to the reaction mixture. The inhibition type was determined by the lineweaver–burk plot, and the inhibition constant was obtained through the secondary plot of the apparent K_m/V_{max} (slope) versus the different concentrations of the apocarotenoids.

Fluorescence quenching studies The experiment was performed as per the protocol of Kim et al. (31) with slight modification. The fluorescence spectra were recorded using a Hitachi F-7000 spectrofluorometer with an excitation wavelength (λ_{ex}) of 290 nm. Briefly, the total reaction mixture containing a constant concentration of 0.77 µM tyrosinase with different concentration of apocarotenoids (10, 25 and 50 µg/ml). The fluorescence quenching data were plotted as the fluorescence intensity against varying concentrations of apocarotenoids. Fluorescence quenching was described by the Stern–Volmer equation (11):

$$F_0/F = 1 + kq\tau_0[Q] = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher respectively, kq is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of the quencher, [Q] is the concentration of the quencher, and K_{SV} is the Stern–Volmer quenching constant.

Hence, the above equation is applied to determine the K_{SV} value using linear regression of a plot of F_0/F against [Q].

Circular dichroism spectroscopy CD spectra were recorded using a Jasco J-715 spectropolarimeter, in a 3-cm rectangular cuvette associated with an optical path length of 1 cm at 25 ± 0.2°C. All spectra were collected three times with a bandwidth of 1.0 nm and a resolution of 0.5 nm at a scan speed of 100 nm/min. The CD measurements for the tyrosinase (0.01 µg of tyrosinase dissolved in 0.1M phosphate buffer, pH 6.8) were obtained after incubation with (50 µg/ml) apocarotenoids at equal molar concentration ratio (1:1). All observed CD spectra were recorded in the wavelength range of 200–250 nm at 37°C under constant nitrogen flush.

Molecular docking studies The molecular docking studies involving tyrosinase and apocarotenoids were carried out through the patchdock server (http://bioinfo3d.cs.tau.ac.il/PatchDock/). The X-ray crystal structure of *Agaricus bisporus* tyrosinase (AbTYR; PDB code, 2Y9W) was chosen as the protein model for our present study (32). The three-dimensional structure of the three apocarotenoids was obtained using CORINA server (https://www.molecular-networks.com/online_demos/corina_demo). The best docking result was selected according to the lowest total score. The figures were obtained through the PyMOL molecular graphics system. The LIGPLOT software was employed to evaluate the docking of apocarotenoids with the amino acid residues of the tyrosinase enzyme (33).

Cell culture B16F0 mouse melanoma cell line was purchased from the National Center for Cell Science, Pune, India. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a humidified atmosphere with a 5% CO_2 incubator at 37°C.

Cell viability assay The cell viability was determined using the MTT assay. The B16F0 cells (5×10^4 cells/well) were cultured in 96 well plate for overnight. The cells were treated for 24 h with different concentrations of apocarotenoids (30, 60, 125, 250, 500 and 1000 µg/ml), which were dissolved in DMSO (0.1% final concentration) and vehicle control (0.1% DMSO). After 24 h treatment, 10 µl of MTT solution (5 mg/ml) was transferred to each well and then incubated for 4 h at 37°C. The formazan crystals were dissolved using 100 µl DMSO and the absorbance was measured at 570 nm using a Biotek ELX-800 microplate reader. Experiments were run in triplicate. Values were expressed as percentage of control.

Cellular melanin content and tyrosinase activity assay Melanin synthesis evaluation and cellular tyrosinase assay was carried out as per the protocol of Pinon et al. (2) with slight modifications. B16F0 cells (1×10^5 cells/ml) were cultured overnight in 24 well plate and then treated with different concentrations of apocarotenoids (30, 60, 125, 250, 500 and 1000 µg/ml) for 24 h. After the incubation period, the cells were lysed in 0.1M sodium phosphate buffer (pH 6.8) containing 0.1% Triton X-100. The lysed cells were then centrifuged at 13,000 rpm for 15 min at 4°C. The obtained melanin pellet was dissolved in 1N NaOH solution containing 20% DMSO at 90°C for 1 h and absorbance was measured at 490 nm using a Biotek ELX-800 microplate reader. The 0.15 ml supernatant containing crude tyrosinase enzyme was added to 0.15 ml of L-DOPA substrate (0.15% in 0.1M sodium phosphate, pH 6.8). The tyrosinase activity was measured at 475 nm using a Biotek ELX-800 microplate reader. The data for melanin synthesis and cellular tyrosinase activity was expressed as percentage of control.

RESULTS

Characterization of bixin The bixin obtained from the seeds of *B. orellana* was characterized through various spectroscopic techniques. The ultraviolet–visible spectroscopy of bixin showed absorbance wavelengths of 502 nm and 470 nm in chloroform solvent, which are the same as the published data of Kovary et al. (21) (Fig. S1A). The FTIR spectra showed peaks corresponding to bixin (Fig. S1B). The broad stretching vibration at 3417 cm^{-1} indicates the presence of –OH group. A sharp aliphatic C–H vibration occurred around 2960–2854 cm^{-1} for the methyl or ethylene group of the compound. The strong absorption bands around 1610–1570 cm^{-1} and 1400 cm^{-1} indicate the carboxylate group of ν_{ass} (COO^-) and ν_{sym} (COO^-) stretching frequencies respectively. The presence of C–O–C stretching vibration was observed at 1161 cm^{-1}. The ^{1}H-NMR spectrum depicted singlet at δ1.8 ppm, which indicates the presence of terminal primary methyl proton. The presence of broad multiplets at δ5.07, 5.82, 6.31, 6.53, 6.64, and 7.12 ppm, corresponding to the methylene protons (Fig. S1C). The ^{1}H-NMR spectrum also exhibited one proton at δ12.35 ppm, ascribed to the acid proton. The ^{13}C-NMR data displayed signals at δ170.43 and 168.01, corresponding to the carbonyl carbons. The methylene carbon signal was observed