INHIBITORY ACTION OF WATER-EXTRACT OF COW SNOUT EPIDERMIS ON MUSHROOM TYROSINASE

YUKIE TAKASHIMA, MASAO HAYASHI, KAZUTAKA TAKEI
AND RYOHEI OGURA

*Department of Medical Biochemistry,
Kurume University School of Medicine,
Kurume, 830, Japan*

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A tyrosinase inhibitor was found in an aqueous extracts of cow snout epidermis, and the possible mode of action to mushroom tyrosinase was investigated. The inhibitor was non-dialyzable, heat-stable and sulfhydryl-containing compound. The sulfhydryl group of inhibitor was essential in the inhibitory activity. The inhibitor combined with the lowest mobility isozyme of tyrosinase on DISC electrophoresis. The growth curve of melanoma B-16 cell was not affected by the culture with inhibitor. The biological role of inhibitor on the skin pigmentation could not be revealed in the present study.

INTRODUCTION

Since the aqueous extracts of mammalian epidermis have been known to inhibit the mushroom tyrosinase, the aqueous extracts contain tyrosinase-inhibitor which is considered in terms of a possible role in controlling skin pigmentation (Flesch, 1949). However, the mechanism of skin darkening on the mammalian epidermis, now appears to be more complicated than it was thought earlier (Fitzpatrick et al, 1966). It is possible that tyrosinase inhibitor may play an active part in the biological and genetical controls which maintain the melanogenesis in melanocyte. In the present paper, the inhibitory effect of the aqueous extract from cow snout epidermis is to be discussed. The purpose of this paper is not to

discuss the biological role in controlling skin darkening, but to find the mode of inhibitory action to the mushroom tyrosinase.

MATERIALS AND METHODS

Material

Cow snout epidermis was used as a source of inhibitor. The cow snout was obtained in a slaughter house. The skin was excised, and the subcutaneous tissue was removed. The epidermis was scraped off with a scalpel, and was lyophilized immediately. The lyophilized material was pulverized in a Wily laboratory mill, to pass through a 40 mesh screen. Powdered material was kept in a freezer until the isolation of inhibitor was performed.

Gel filtration of crude extract

Ten mg of powdered epidermis was homogenized in a glass homogenizer with 10 ml of 0.1 M phosphate buffer (pH 6.8), and kept stirring in cold room for 60 minutes. The homogenate was centrifuged at $600 \times g$ for 15 minutes, and the supernatant was filtered through three folds of a gauge to remove an aggregates of lipids. The resulting filtrate was used as the crude extract. Routinely 5 ml of crude extract was applied to a column of Sephadex G-25 (2.5×55 cm) equilibrated with 0.1 M phosphate buffer (pH 6.8), and the column was eluted with same phosphate buffer. The effluents from the column were collected in 5 g fractions after passing through a UV monitoring device for continuously assaying the absorption of effluent at 280 nm.

Tyrosinase assay

Mushroom tyrosinase (O-diphenol: O₂ oxidoreductase EC. 1. 10. 31.) was purchased from Nutritional Biochemical Corporation (1,050 units/1 mg, II grade). Tyrosinase activity was determined by the spectrophotometric method. The standard reaction mixture, consisting of 0.27 ml of 1.77 mM L-tyrosinase, 0.03 ml of 1.77 mM L-dopa, 0.20 ml of 0.5 M phosphate buffer (pH 6.8) and 1.30 ml of distilled water, was pipetted into a quartz cell placed in a constant temperature cell holder (37°C) of a Beckman DU spectrophotometer. The change in optical density (OD) at 475 nm was recorded for 5 minutes, following addition of 0.2 ml of 1 mg/ml mushroom tyrosinase to the reaction mixture. Under above condition, the increase rate in optical density was linear with time up to 10 minutes incubation. Tyrosinase activity was expressed as the change

of optical density per one minutes at 37°C in above condition. In a simultaneous experiment the distilled water in the reaction mixture was replaced by aliquot of inhibitor, and tyrosinase activity was determined. The inhibitory activity was expressed as the percentage of decrease of tyrosinase activity in the reaction mixture, compared to control.

Determination of sulfhydryl

For the determination of sulfhydryl group (SH) in protein, the amperometric titration technic using the rotating platinum electrode was applied (Kolthoff et al., 1965).

Protein concentration

Protein concentration was determined by the procedure of Lowry et al., with crystalline bovine serum albumin as standard (Lowry et al., 1951)

Acrylamide disc electrophoresis

Disc electrophoresis was performed with 7.5 % polyacrylamide gel according to that of Davis (1964). Electrophoresis of mushroom tyrosinase (0.1 ml of 0.1 % solution) was run in 0.02 M Tris-buffer solution pH 8.3. All gels run at 5°C at a constant current (3 mA per gel) were soaked in 0.5 M buffer (pH 6.5) for 30 minutes in room temperature to equilibrate the acrylamide gels with the buffer pH 6.5. And the gels were incubated 40 minutes at 37°C in a 0.15 % dopa-0.1 M phosphate buffer solution to develop the full color intensity following dopa-tyrosinase reaction. The gels stained with dopa-melanin were then scanned by transmittance in a ATAGO Densitometer by means of a 440 nm filter.

RESULTS

Comparison of skins from different area

The crude extracts (2 mg dry weight of epidermis) from black and white areas of the cow snout epidermis were compared with the inhibitory activity. In the cases of the white skin, a higher inhibition of initial velocity and delay of lag phase were observed.

Enzyme kinetics

The initial velocity of mushroom tyrosinase activity was determined at the various concentrations of substrate, with a fixed amount of crude extract. The reaction is slowed considerably at low substrate concentration.

The modifier has less effect, as more substrate is added. As shown in Fig. 1, sigmoid saturation curve for substrate was obtained in the presence of crude extract. The inhibition to the enzyme-catalytic reaction was determined using the method of Lineweaver-Burk plot (Fig. 2). The effect of inhibitor was concluded as non-competitive inhibition, because the crude inhibitor decreased V_{max} and did not alter K_m value (K_m : 1.0μ mole).

Chemical properties of inhibitor

The crude extract was subjected to various tests to find some of the chemical characteristics of the inhibitor.

a) Urea treatment

One ml of crude extract was treated with 1 ml of 8 M urea for 1 hour, and then determined the inhibition to mushroom tyrosinase. The crude inhibitor lost considerably its inhibitory activity.

Fig. 1 Correlation between initial velocity and concentration of substrate.

Fig. 2 Non-competitive inhibition on dopachrome formation catalyzed by mushroom tyrosinase (LINEWEAVER-BURK).

b) Trichloroacetic acid (TCA) treatment

One ml of 5 % TCA solution was added to 1 ml of crude extract. The

precipitate was washed with 2.5 % TCA solution several times, and was resuspended in 1 ml distilled water. The pH of both supernatant and resuspended precipitate was adjusted to 7.2, and each of them was determined for inhibitory activity. Less remaining activity was detected in the supernatant.

c) Thermolability

The crude extract (dry weight 10 mg per ml) was maintained at 100 °C for the desired period of times. The heat-denatured protein was removed by centrifugation. The inhibitory activity remained in supernatant was determined. Forty per cent of inhibitory activity retains in supernatant after 30 minutes heat-treatment at 100 °C. After treatment above 60 minutes the crude extract lost all its inhibitory activity. The thermolability of crude extract is shown in Fig. 3.

d) Dialysis

Five ml of crude extract was dialyzed

Fig. 3 Thermolability of inhibitor.

against distilled water for 24 hours at 4°C. The diffusate was concentrated to 5 ml by lyophilization. The concentrated diffusate and dialysate were determined for their ability of inhibition. The inhibitory activity was less in the diffusate.

Fig. 4 Sephadex G-25 column gel filtration of crude extract.

TABLE 1
*Inhibitory activity of various fractions obtained
 from sephadex G-25 gel filtration of crude extract*

Fraction	Total protein	Total SH	SH per mg protein	Tyrosinase activity	Inhibition
Control				0.31 OD/min	
0-5 Tubes	—	—	—	0.27	(—)
I	0.39 mg	0.46 μ g	0.12 μ g	0.29	(—)
II	2.46	3.56	1.45	0.21	(+)
III	0.40	—	—	0.31	(—)
IV	—	—	—	0.30	(—)
44 ~ Tubes	—	—	—	0.28	(—)

Partial purification of crude extract

Gel filtration of crude extract on Sephadex G-25 gave four fractions, as shown in Fig. 4. Each fraction was concentrated by lyophilization, and was determined the inhibitory activity to mushroom tyrosinase. The inhibitory activity was found in the 2nd fraction as shown in Table 1.

In the additional experiment, elution position of glutathione (GSH) emerging from the column applied to the Sephadex 25, corresponds to the 3rd fraction. It was confirmed chromatographically, that the inhibitor is not equivalent to glutathione. The 2nd fraction was then lyophilized, and was used as a partially purified inhibitor in the following experiment.

TABLE 2
Aging and inhibitory activity

Material	Inhibitory activity	SH content
Fresh material	32.3 %	1.45 μ g
Aging material (1)	0.03	0.12
(2)	None	Negligible

SH content is expressed as μ g per mg protein.

Effect of aging

The crude extract was able to keep in a refrigerator for 30 hours without any loss of activity. However, the partially purified inhibitor lost its ability of inhibition unless it was lyophilized. The partially purified inhibitor contains 1.45 μ g of SH per mg protein, but SH group was not detected in the aging inhibitor, as shown in Table 2. The aging resulted in a loss of activity and was accompanied with an oxidation of sulfhydryl groups.

The partially purified inhibitor was further reacted with p-chloromercuribenzenate (PCMB), which blocks free SH group in inhibitor. The reacted mixture was applied to the sephadex G-25 column to separate the PCMB-bound inhibitor from the excess of PCMB solution. The PCMB-bound inhibitor was found to contain no free sulfhydryl and to have no ability of inhibition. The PCMB-bound inhibitor was treated again with β -mercaptoethanol for reducing the PCMB-bound SH to free SH group. The mercaptoethanol-treated inhibitor could restore the ability of activity (Table 3).

It was assumed, therefore, that sul-

thiyl groups play an important role in the activity of inhibitor.

Disc electrophoresis

To study the interaction between mushroom tyrosinase and inhibitor, the three mixtures, A (mushroom tyrosinase alone), B (with both tyrosinase and purified inhibitor), and C (with both tyrosinase and PCMB-treated inhibitor) were applied to the Disc electrophoresis respectively. The Disc-gel

TABLE 3

Effect of p-chloromercuribenzoate treatment to the inhibitory activity of inhibitor

Material	SH content	Inhibitory activity
Inhibitor	1.45 μ g/mg	32.7%
PCMB-bound inhibitor	None	None
Mercaptoethanol treated inhibitor		31.7%

Fig. 5 Densitogram of mushroom tyrosinase on the disc electrophoresis.

was stained with dopa-tyrosinase reaction as described before. The densitogram of mushroom tyrosinase (A) showed four prominent isozyme bands as shown in Fig. 5-A.

However, the B-mixture of tyrosinase and inhibitor showed the three bands on the densitogram. The reaction of tyrosinase with inhibitor resulted in the disappearance of the 4th isozyme band which has the lowest mobility on Disc electrophoresis (Fig. 5-B). The tyrosinase which was treated with PCMB-bound inhibitor, was applied to

Disc electrophoresis (Fig. 5-B). The Densitogram showed the original four isozyme bands without any disappearance of the band. It is concluded that the partially purified inhibitor combines with the lowest mobility isozyme of mushroom tyrosinase through sulfhydryl group.

Effect of tyrosinase inhibitor to melanoma cell growth

Mouse melanoma B-16 was used as cell line for tissue culture. Approxi-

mately 2×10^4 of cell was inoculated in Eagle minimum essential medium supplemented with 15 % chiba calf serum placed in the ϕ 35 mm plastic dish, and was incubated at 37°C . After incubation of 5 hours, 1 ml of partially purified inhibitor, or, distilled water as control was added to the tissue culture dish. The concentration of inhibitor added in dish was 1.75 mg and 0.35 mg in protein respectively. The inhibitor with 1.75 mg in protein concentration could decrease the mushroom tyrosinase to 60 % in activity (40% of inhibition). The growth curve of B-16 cell was observed for 72 hours. As shown in Fig. 6, the significant difference was not detected in each growth curve of B-16 cultured cell.

Fig. 6 Effect of tyrosinase inhibitor to growth curve on melanoma B-16.

In the separate experiment, 1 ml of purified inhibitor (1.75 mg in protein concentration) or distilled water as control, and 0.3 ml of ^3H -dopa ($0.3 \mu\text{Ci/ml}$) was added to B-16 tissue culture dish, which was cultured previously for 5 hours in ϕ 60 mm plastic dish inoculated with 5×10^4 cell. After incubation for 48 hours the cultured cells were

trypsinized, and the cells were thoroughly washed with phosphate-buffer-saline solution (pH 7.4). Ten % trichloroacetic acid was added to the washed cell, and the precipitate was washed trichloroacetic acid several times. The radioactivity of acid insoluble fraction was determined by liquid scintillation counter. As shown in Table 4, the presence of inhibitor caused the remarkable decrease on the ^3H -dopa incorporation into the acid insoluble fraction of melanoma B-16. However, the PCMB-bound inhibitor did not show any decrease in the incorporation.

The partially purified inhibitor with the concentration which did not show any inhibition on the growth of B-16 cultured cells, caused a significant decrease of ^3H -dopa incorporation into the melanoprotein of B-16 cell.

TABLE 4

Effect of tyrosinase inhibitor to the incorporation of ^3H -L-DOPA into melanoma B-16 cell

	Total count of ^3H -L-DOPA in reaction mixture	Incorporation of ^3H -L-DOPA into the acid insoluble fraction
Control	3640	1160 cpm
Tyrosinase inhibitor	4320	480
PCMB-bound inhibitor	3720	1080

DISCUSSION

Early studies of the inhibitor obtained from human epidermis (Rothman et al., 1964 ; Flesch, 1949), suggested the presence of dialyzable, water soluble, SH-containing compound which inhibited the formation of melanin from tyrosine

TABLE 5

Property of inhibitor isolated from mammalian skin

Source of material	Nature of material	Reference
Human skin	Water soluble, heat stable dialyzable nonprotein SH	FLESCH ROTHMAN 1946, 1948
Human, mouse skin	Two fractions : Heat labile, SH heat stable, non SH	QUEVEDO 1960
Guinea pig skin	Water soluble, heat stable non-dialyzable, UV stable non-SH protein	FLAWN WILDE 1970
Cow snout skin	Water soluble, heat stable, non dialyzable	OGURA 1970

and dopa by mushroom tyrosinase. The presence of a tyrosinase inhibitory factors in other mammalian skins has been reported. Some properties of inhibitors are summarized in Table 5.

The properties of inhibitor may be divided into two main types, those described as heat-stable, dialyzable, non-protein, and those described as non-dialysable, protein compound. In our present experiment, the inhibitor from cow snout epidermis was heat-stable, non-dialyzable, and was relatively small molecular weight compound containing sulfhydryl group.

The inhibitor in the aqueous extracts from human epidermis reported by Rothman and Flesch (1946, 1949), was considered to be a reduced glutathione in the epidermis. Halprin et al. (1966) have described that glutathione in the epidermis exists over 90% in the reduced form, although reduced form is constantly being oxidized within the cell. NADPH-dependent glutathione reductase is found to be essential for recovering oxidized glutathione to reduced form. Seiji et al. (1969) have studied that glutathione binds to one of the oxidation products of substrate, and thus preventing its further oxidation.

It was assumed that the inhibitory effect of thiol compounds on melanin formation in the melanocyte is mainly due to the formation of thiol-derived reaction products and not the inhibition of tyrosinase.

In contrast, Quevedo (1960), Flawn et al. (1970) have found the inhibitor to be a non-dialyzable non-sulfhydryl compound-probably proteinaceous.

Most workers have used whole skin as the source of inhibitor, and have not tried to locate the inhibition further. The inhibitor from guinea pig skin described by Ginsberg (1944) was restricted to the epidermis. Flawn et al. (1970) have also described the natural inhibitor of dopa autoxidation from guinea pig epidermis. The inhibitor was not dependent upon the presence of sulfhydryl groups, and was heat and UV-stable. This active compound was a protein of molecular weight about 6,000 and prevented the oxidation of tyrosine to melanin.

In our present paper, the partially purified compound is a non-competitive inhibitor of mushroom tyrosinase. It inhibits the mushroom tyrosinase by combining with the one of tyrosinase isozyme. Sulfhydryl group is essential

in activity. On the typical acrylamide gel electrophoresis the pattern of mushroom tyrosinase consists of four prominent bands, numbered I, II, III and V, (Jolley, 1967). The band originally designated as IV does not appear in most preparations. The interconversion study of isozymes by the work of Jolley (1967) has shown that Band III (tetramer) is readily dissociated at 50 °C to Band II and Band I (monomer). Band V has found to be a higher polymer than the Band III tetramer. It is interesting that the Band V is inactivated rapidly following preincubation with our partially purified inhibitor. Band I, II and III display great resistant to activity of partially purified inhibitor.

The inhibitor is not only capable of causing inhibition of mushroom tyrosinase, but decreases the incorporation of ^3H -dopa into the B-16 melanoprotein. But the PCMB-bound inhibitor did not show any effect on the tyrosinase activity and incorporation of ^3H -dopa. The growth of melanoma B-16 cultured cell was not effected under the presence of inhibitor which caused the decrease of substrate-incorporation and tyrosinase activity.

It can be seen that in the case of the white skin a strong inhibition of mushroom tyrosinase was observed. However, the role of this material in skin pigmentation and genetic control has not been resolved. It is not identical whether the partially purified material is natural inhibitor reducing melanin biosynthesis in melanocyte, or happens to inhibit the mushroom tyrosinase in the *in vitro* experiment.

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REFERENCES

- DAVIS, B. (1964). Disc electrophoresis. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 217-222, 404-427.
- FITZPATRICK, T.B., MIYAMOTO, M. and ISHIKAWA, K. (1966). The evolution of concepts of melanin biology. *Advances in Biology of skin*, (W. Montagna and F. Hu, ed.) vol. VIII, pp. 1-39. Pergamon Press, New York.
- FLAWN, P.C., BIOL, M.I. and WILDE, P.F. (1970). Isolation and partial characterization of the natural inhibitor of dopa autoxidation in skin. *J. Invest. Derm.*, **55**, 153-163.
- FRESCH, P. (1949). Inhibitory action of extracts of mammalian skin on pigment formation. *Proc. Soc. exp. Biol. Med.*, **70**, 136-137.
- GINSBERG, B. (1944). The effect of the major genes controlling coat colour in the guinea pig on the dopa oxidase activity of skin extracts. *Genetics*, **29**, 176-198.
- HALPRIN, K.M. and OHKAWARA, A. (1967). The measurement of glutathione in human epidermis using glutathione reductase. *J. Invest. Derm.*, **48**, 149-152.
- HIRSCH, H.M. (1955). Tissue autoxidation inhibitors 1. The inhibition of dopa autoxidation by extracts from normal and neoplastic tissues. *Cancer Res.*, **15**, 249-255.
- JOLLEY, R.L. (1967). The tyrosinase isozymes. *Advances in Biology of skin*, (W. Montagna and F. Hu ed.) Vol. VIII, pp. 269-281. Pergamon Press, Ltd., New York.
- KOLTHOFF, I.M., SHORE, W.S., TAN, B.H. and MATSUOKA, M. (1965). A critical study of amperometric titration methods for sulfhydryl determination in native and denatured bovine serum albumin. *Anal. Chem.*, **12**, 497-508.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**, 256-275.
- QUEVEDO, W.C. and ISHERWOOD, J.E. (1960). Mammalian pigmentation. *J. Invest. Derm.*, **34**, 309-315.
- SEIJI, M., YOSHIDA, T., ITAKURA, H. and IRIMAJIRI, T. (1969). Inhibition of melanin formation by sulfhydryl compounds. *J. Invest. Derm.*, **52**, 280-286.