ACTIVATION OF EPIDERMAL TYROSINASE

Joseph S. McGuire
Yale University School of Medicine
New Haven, Connecticut 06510

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SUMMARY: A soluble fraction obtained from the epidermis of Rana pipiens contains tyrosinase which either is competitively inhibited or is present as protyrosinase. Exposure of the epidermal preparation to trypsin or chymotrypsin activates the tyrosinase. There is no demonstrable activity before exposure to trypsin; after activation, dorsal (pigmented) epidermis contains roughly as much tyrosinase as ventral (white) epidermis. The activation of protyrosinase by protease offers a new site for the control of pigmentation.

Tyrosinase activity in frog (Rana pipiens) skin was first demonstrated in 1957 by Purvis and Denstedt (1) who found that exposure of the skin to trypsin or chymotrypsin "solubilized" the enzyme permitting its release. Recently, in a series of experiments designed to demonstrate a relationship between the level of frog epidermal tyrosinase and treatment with melanocyte-stimulating hormine, the extraction of tyrosinase from frog skin was re-examined. Tyrosinase has two catalytic functions: the oxidation of tyrosine to dihydroxy-phenylalanine (DOPA) and the oxidation of DOPA. The former activity was measured by the technique of Pomerantz (2). In this assay, $^{3}H_2O$ produced by the oxidation of 3, 5, ^{3}H-tyrosine is collected and measured in a liquid scintillation spectrometer. The latter activity (DOPA oxidase) was monitored by measuring the increase in absorbancy at 475 nm associated with the formation of DOPA-chrome from DOPA (3).

In this report, evidence for an inactive form of tyrosinase in frog epidermis and its activation either by trypsin or chymotrypsin will be presented. Frogs (Rana pipiens) were decapitated and pithed; the dorsal and ventral skin was removed and washed in 0.1 M. sodium phosphate buffer pH 7. The epidermis was removed manually after the skin had been soaked in 2 M. NaBr for 1.5 hours. The epidermis was homogenized in 0.1 M. PO_4 buffer pH 7 and the homogenate was centrifuged further as indicated in the legends. Purvis and Denstedt (1) added trypsin directly to frog skin before homogenizing it. In order to eliminate

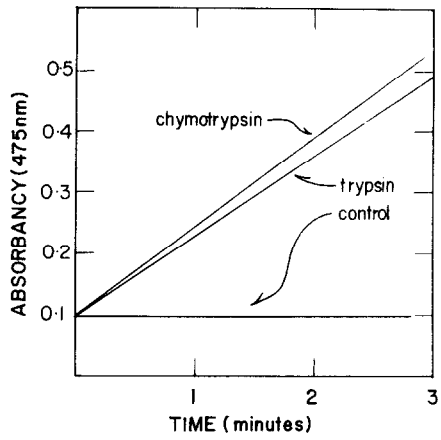

Figure 1. Activation of DOPA oxidase by trypsin or chymotrypsin. The oxidation of L-DOPA to DOPA-chrome was measured in a Gilford multiple sample absorbance recorder at 475 nm according to the method of Fling et al.[3] Cuvettes were read at 5 second intervals. Each cuvette contained 0.5 ml sodium phosphate buffer pH 7, 0.1 M.; enzyme, 10 µl; L-DOPA 2 mg/ml phosphate buffer, 0.4 ml; and, where indicated, trypsin or chymotrypsin 10 µl. The total volume of the reaction mixture was 0.92 ml. The enzyme was prepared from frog epidermis separated from dermis after 1.5 hr. exposure to 2 M. NaBr at room temperature. The epidermis was homogenized in 0.1 M. PO_4 buffer and centrifuged at 27,000 x g. The supernatant fraction was further centrifuged at 200,000 x g and this supernatant was dialyzed at room temperature against 0.05 M. PO_4 buffer pH 7 for four hours. The enzyme was lyophilized in 5 ml aliquots which were reconstituted with 1 ml distilled water just before use. The enzyme thus reconstituted contained 22 mg protein/ml; 10 µl equivalent to 220 µg protein was added to each cuvette. Buffer, enzyme, and, where indicated, trypsin or chymotrypsin were mixed in the cuvette and allowed to sit two minutes. DOPA was added; the contents again were mixed and the cuvettes were placed in the spectrophotometer. The rate of increase in absorbancy was linear for approximately four minutes.

Cuvette 1 (control) contained buffer, DOPA and enzyme, and there was no increase in absorbancy (DOPA was not oxidized to DOPA-chrome).

Cuvette 2 (trypsin) contained the reaction mixture in cuvette 1 plus 100 µg TPCK-trypsin which is trypsin obtained from Worthington that has been treated with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone to inhibit chymotryptic activity. DOPA was rapidly oxidized.

Cuvette 3 (chymotrypsin) contained the reaction mixture in cuvette 1 with the addition of 0.78 µg chymotrypsin (α-chymotrypsin, 3 x crystallized, obtained from Worthington). The presence of chymotrypsin permitted the rapid oxidation of DOPA to DOPA-chrome.

the possibility that trypsin was simply solubilizing the cell allowing the release of tyrosinase, a high speed supernatant fraction was studied. A 27,000 x g supernatant was further centrifuged at 200,000 x g for 60 minutes in a Spinco Model L ultracentrifuge. The 200,000 x g supernatant did not oxidize either tyrosine or DOPA; however, the addition of small amounts of either trypsin or chymotrypsin to this supernatant fraction permitted the rapid oxidation of these substrates. (Fig. 1) The data in this report were obtained with preparations of mixed dorsal and ventral epidermis; however, when dorsal (pigmented) and ventral (white) epidermis were examined separately, they were found to contain

Table I. Activation of tyrosinase by trypsin.

	TYROSINASE (CPM ^3H_2O RELEASED)	
	WITHOUT TRYPSIN	WITH TRYPSIN
KIDNEY	134	145
INTESTINE	161	144
LIVER	181	147
EPIDERMIS	155	9,026
BLANK (TISSUE OMITTED)	196	

^3H_2O released from tyrosine 3, 5, ^3H was collected and measured according to the method of Pomerantz (2). 1 ml of the 25 ml collection from the column was counted. Each organ was homogenized in 5 ml of 0.1 M. PO_4 buffer pH 6.8 and centrifuged at 34,000 x g for one hour. The reactions were incubated for 15 min. at 37°. Each reaction mixture contained in a total volume of 2.5 ml, enzyme 0.5 ml, buffer (PO_4, pH 6.8, 0.1 M.) 1 ml, where indicated, 1 mg trypsin (Difco 1:250) in 50 µl buffer, and 1 ml L-tyrosine-3, 5, ^3H. The labelled tyrosine was prepared by mixing 100 µ moles of unlabelled L-tyrosine with 3×10^{-3} µ moles labelled L-tyrosine (sp. act. 35 c/m mole). The tyrosine was recrystallized from water, washed 2x by centrifugation, dried, weighed, and redissolved in water to give a 1.75×10^{-3} M. solution.

comparable amounts of trypsin-activable tyrosinase. This dramatic activation of tyrosinase was not demonstrable in other tissues (Table I).

Stimulation of tyrosinase by trypsin could be prevented by prior addition of crystalline soybean trypsin inhibitor (Fig. 2). The addition of trypsin

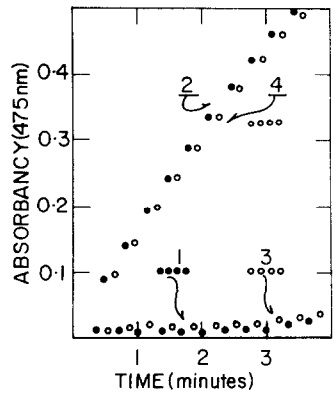

Figure 2. Effect of soybean trypsin-inhibitor on the activation of frog enzyme by trypsin.
The oxidation of DOPA to DOPA-chrome was monitored at 475 nm as in Fig. 1 in a Gilford multiple sample absorbance recorder.

Cuvette 1 contained 0.5 ml phosphate buffer pH 7.0, 0.1 M. 10 µl frog epidermal enzyme (220 µg protein), and 0.4 ml DOPA (2 mg l-dihydroxyphenylalanine/ml phosphate buffer). DOPA was not oxidized.

Cuvette 2 contained the reaction mixture in cuvette 1; however, 100 µg TPCK-trypsin was added to the buffer-enzyme mixture 2 minutes before the addition of DOPA.

Cuvette 3 contained the reaction mixture in cuvette 2. Before the addition of TPCK-trypsin, 200 µg crystalline soybean trypsin-inhibitor was added. The prior addition of trypsin-inhibitor prevented the activation of DOPA-oxidase by TPCK-trypsin.

Cuvette 4 contained the reaction mixture in cuvette 2. Two minutes after the addition of TPCK-trypsin, but before the addition of DOPA, trypsin inhibitor was added. The rate of DOPA-chrome production was the same as observed in cuvette 2 to which no trypsin inhibitor had been added.

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inhibitor to the reaction mixture two minutes after the addition of trypsin did not influence the activation of tyrosinase. Activation of tyrosinase with 100 µg TPCK-trypsin was complete in less than two minutes. If 5 µg TPCK-trypsin was used, the time for half-activation was four minutes.

Appearance of tyrosinase activity in the 200,000 x g supernatant fraction following addition of these endopeptidases might be attributed to the destruction of an inhibitor. Evidence against such an explanation is the observation that dialysing the 200,000 x g supernatant for 4 hours against 0.05 M. PO_4 buffer pH 7.0 and then passing it through a 50 cm Sephadex G-100 column did not

"uncover" tyrosinase activity, which could, at this step in the preparation be activated by either trypsin or chymotrypsin. Both the "proenzyme" and activated enzyme were unretarded when chromatographed on a 50 cm Sephadex G-100 column and only slightly retarded by a G-200 column. This suggests that the enzyme is large and that a relatively small fragment or inhibitor is removed by trypsin.

If excess inhibitor were present in the unactivated preparation, it might be expected to inhibit trypsin-activated tyrosinase; however, the addition of unactivated enzyme to enzyme already activated by trypsin did not reduce the activity of the latter. (Fig. 3).

These observations support the notion that tyrosinase, easily extracted from frog epidermis in a form readily activated by endopeptidases, probably exists within the epidermis as a proenzyme. The findings can also be explained

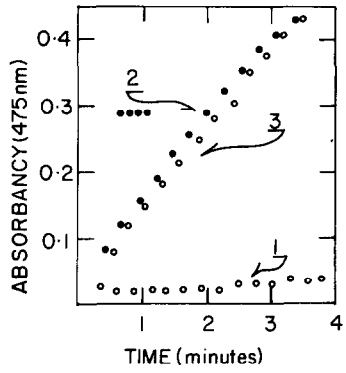

Figure 3. Lack of inhibition of previously activated enzyme by the addition of unactivated enzyme.
The oxidation of DOPA to DOPA-chrome was monitored at 475 nm in a Gilford multiple sample absorbance recorder as described in Fig. 1.

Cuvette 1 contained PO_4 buffer 0.1 M., pH 7, 0.5 ml; frog epidermal enzyme 10 µl (220 µg protein), and DOPA 0.4 ml.
Cuvette 2 contained the reaction mixture of cuvette 1; however, 100 µg of TPCK-trypsin and, two minutes later, 200 µg crystalline trypsin soybean inhibitor was added before the addition of DOPA.
Cuvette 3 contained the reaction mixture in cuvette 2, however, before DOPA was added, an additional 10 µl of frog epidermal enzyme was added. The rate of DOPA oxidation in this reaction was identical to that in cuvette 2 indicating that the unactivated epidermal enzyme did not inhibit the trypsin-activated enzyme.

by an inhibitor associated with tyrosinase tightly enough to withstand both dialysis and sephadex chromatography. Trypsin or chymotrypsin might destroy such an inhibitor or its binding to tyrosinase. Epidermal melanocytes are abundant in the dorsal epidermis of the frog. Melanocytes have not yet been demonstrated in ventral epidermis although dendritic non-melanized cells are present (unpublished observations). The roughly equal distribution of "protyrosinase" in dorsal and ventral epidermis suggests that either the enzyme is not of melanocytic origin or that under in vivo conditions protyrosinase is not activated in occult ventral epidermal melanocytes.

The characteristics of frog epidermal tyrosinase described here may help explain past difficulties in demonstrating mammalian epidermal tyrosinase. The activation of mammalian tyrosinase by ultraviolet light has been attributed to the dissociation or destruction of a tyrosinase inhibitor (4). The observations on frog tyrosinase permit the speculation that cathepsins released by ultraviolet irradiation activate tyrosinase. This possibility is being approached experimentally.

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References

1. Purvis, J. L., Denstedt, O. F. (1957). Canad. J. Biochem. Physiol., 35, 961.
2. Pomerantz, S. H. (1966). J. Biol. Chem., 241, 161.
3. Fling, M., Horowitz, N. H., Heinemann (1963), J. Biol. Chem. 238, 2045.
4. Fitzpatrick, T. B., Becker, S. W., Jr., Lerner, A. B. and Montgomery, H. (1950), Science, 112, 223.