The Tyrosinase–Protyrosinase System in Frog Epidermis

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INTRODUCTION

The formation of melanin in vertebrate skin has been the subject of controversy since Bloch found that black pigment was formed in melanocytes when human skin was incubated with dopa (dihydroxyphenylalanine). No such pigment was formed when the skin was incubated with tyrosine or epinephrine. (1) In view of subsequent observations that extracts of human and mouse melanomas can oxidize tyrosine as well as dopa, Lerner recommended that the single term tyrosinase be used to describe the enzyme responsible for catalyzing the oxidation of tyrosine and dopa. (2) The demonstration of tyrosine conversion to melanin by mammalian skin required special effort. It was not until 1950 that Fitzpatrick et al. irradiated human volunteers with ultraviolet light for 8 days and found that slices of skin incubated in tyrosine became dark. Microscopically, dark granules were found in the cytoplasm of the cell body and dendritic processes of the melanocytes. The interpretation of these observations was that tyrosinase does exist in human epidermis but in an inactive or partially inhibited state. (3)

Several years ago in experiments designed to study the induction of frog skin tyrosinase by melanocyte-stimulating hormone, proteolytic enzymes were found to activate epidermal tyrosinase of Rana pipiens. The nature of the activation suggested a proenzyme–enzyme conversion. (4)

The inactive tyrosinase has been purified and compared with active tyrosinase produced by chymotrypsin proteolysis. In this report, we conclude that the inactive tyrosinase is a true proenzyme and that activation consists of the proteolytic cleavage of a low molecular weight peptide probably from the N-terminal portion of the molecule.

The active enzyme possesses both tyrosinase and dopa oxidase functions that remain in constant relationship throughout purification. No evidence for participation of peroxidase could be found in the oxidation of either tyrosine or dopa.

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MATERIALS

*Rana pipiens* were obtained from Mogul-Ed, Oshkosh, Wisconsin. L-[3,5-3H] tyrosine (36 Ci/mM) was purchased from Amersham/Searle.

Carboxymethyl cellulose CM 52 and Sepharose 6B were products of H. Reeve Angel, Clifton, New Jersey, and Pharmacia Fine Chemicals, Piscataway, New Jersey, respectively. α-Chymotrypsin (bovine pancreatic) and trypsin inhibitor (soybean) were obtained from Sigma. Other enzymes and proteins were purchased from Sigma, Worthington, or Calbiochem. Glass-distilled water was used to prepare all solutions.

METHODS

Assays. Tyrosine hydroxylation was measured at 25°C by determining the formation of 3H₂O from [3,5-3H] tyrosine according to the technique of Pomerantz (5). Tyrosine and dopa concentrations were, respectively, 5 × 10⁻⁴ M and 2.5 × 10⁻⁵ M in a 1 ml reaction volume of 0.1 M Na phosphate buffer, pH 6.8. A unit of enzymic activity is defined as 1 μmole of L-tyrosine oxidized per minute.

Enzymic activity was determined spectrophotometrically at 25°C using L-dopa as substrate as suggested by Fling *et al.* (6). The reaction mixture consisted of 1 ml of 5 mM L-dopa in 0.1 sodium phosphate pH 6.8. The molar absorbancy at 475 nm for dopa-chrome was assumed to be 3600 cm⁻¹ (7) and a unit of enzymic activity was defined as 1 μmole of dopa-chrome produced per minute. The proenzyme was activated by adding 10μl of chymotrypsin 5 mg/ml to the enzyme contained in the assay cuvette, incubating 3 min at 25°C, and stopping the reaction by addition of 20μl of soybean trypsin inhibitor 20 mg/ml. Active enzyme was prepared in quantity by adding to purified proenzyme 2% chymotrypsin by weight and incubating at 37°C until maximum activity developed. Proteolysis was stopped, if necessary, by addition of soybean trypsin inhibitor, N-tosylphenylalanyl chloromethylketone, or diisopropylfluorophosphate.

Amino acid analysis. Amino acid analyses were performed by Dr. William Konigsberg on 0.2 mg samples of pure proenzyme hydrolyzed in 6 N HCl for 24 and 48 hr in evacuated tubes.

Protein measurement. Routine determinations of proenzyme protein were performed spectrophotometrically using an extinction coefficient ε₆₅₀⁻¹⁹⁺ = 15.0 cm⁻¹, estimated from the amino acid analysis of the material. The method of Lowry *et al.* (8) was used on samples too turbid to permit such analysis.

Polyacrylamide gel electrophoresis (PAGE). Acrylamide and N,N'-bisacrylamide were purified before use by recrystallization. Polypeptide molecular weights were determined by the SDS gel procedure of Weber and Osborn (9). Protyrosinase and tyrosinase were studied on acrylamide gels containing 9.8% acrylamide and 0.17% bisacrylamide; while the small peptide produced by activation was electrophoresed on a gel consisting of 14.2% acrylamide and 0.77% bisacrylamide.

Analytical PAGE of the intact enzyme was performed in a continuous gel system of 4.8% acrylamide and 0.25% bisacrylamide. The running buffer was 0.025 M bis-tris acetate, pH 6.0. Samples were applied in a 20-fold dilution of this medium. Under these conditions the enzyme migrates toward the cathode, and crystal violet is a suitable tracking dye. Enzymic activity was visualized on the gels by treatment with 10 mM dopa in 0.1 M sodium phosphate, pH 7.0 containing, for proenzyme samples, 0.01 mg/ml chymotrypsin. Protein was stained with Coomassie Blue 6B.
Analytical gel filtration. The molecular weights of proenzyme and active enzyme were estimated by gel filtration through a 100 × 1.2 cm column of Sepharose 6B.

Copper Analysis. Copper was determined by atomic absorption analysis through the courtesy of Dr. J. E. Coleman. A Jarell-Ash spectrometer was employed at 3247 Å using the "spike-height" method of absorbance determination (10).

Sucrose density gradient centrifugation. Sedimentation coefficients for the enzymes were evaluated in a Beckman Model L ultracentrifuge by the sucrose density technique of Martin and Ames (11). Bovine catalase was used as an internal standard with an assumed \( S_{20,w} = 11.3 \text{s} \).

Isoelectric focusing. Enzyme samples were subjected to isoelectric focusing at 4°C and 600 V on a pH 7–10 Ampholine gradient for 90 hr. A 110-ml capacity apparatus from LBK Instruments was employed.

RESULTS

Purification of Enzyme

The purification was simplified by the basicity of frog epidermal tyrosinase (Table 1). Both the proenzyme and the activated protein are adsorbed by carboxymethyl cellulose under conditions where few other proteins bind. Separation of frog skins and chromatography over CMC were performed at room temperature; all other steps, at 4°C. Buffer was, unless otherwise stated, an equimolar mixture of mono- and disodium phosphates, pH 6.8, diluted to the appropriate concentration.

Extraction of tyrosinase. The skins from 30 to 40 frogs were soaked for 1 hr in 2 M KBr; the epidermis was removed from the dermis and placed in 0.1 M Tris–HCl, pH 7.5. The tissue was homogenized in a motor-driven Potter-Elvehjem apparatus. The extract was centrifuged 20 min at 40,000 g at 4°C and \( \frac{1}{2} \times 0 \), vol 1 M MnSO₄ was added to the supernatant to precipitate nucleic acids (12). Centrifugation for 1 hr at 100,000 g removed the manganese precipitate together with subcellular material.

Ammonium sulfate fractionation. The tyrosinase was isolated as the 30–65% saturated (NH₄)₂SO₄ fraction. The pellet was dissolved in a minimal volume of 0.1 M Na Phosphate buffer, pH 6.8.

| Fraction                          | Volume (ml) | Total protein (mg) | Dopa-oxidase activity | Tyrosine-hydroxylase activity |
|-----------------------------------|-------------|--------------------|------------------------|-----------------------------|
|                                   |             |                    | Total (units)          | Specific (units/mg)         | Total (units) | Specific (units/mg) | Yield (%) |
| Epidermal homogenate              | 89          | 565                | 294                    | 0.52                        | 43.6          | 0.077               | —         |
| MnSO₄ supernatant                 | 92          | 586                | 285                    | 0.48                        | 42.2          | 0.075               | 97.       |
| 30–65% (NH₄)₂SO₄ precipitate      | 11.2        | 244                | 265                    | 1.19                        | 42.8          | 0.191               | 90.       |
| Redissolved precipitate after dialysis | 19.0    | 184                | 236                    | 1.28                        | 26.4          | 0.143               | 80.       |
| CMC Eluate                        | 6.1         | 1.89               | 67                     | 35.4                        | 10.9          | 5.77                | 23.       |

* The data is from a representative preparation of 34 frogs. Yields are calculated from the recovery of dopa oxidase activity. The ratio of dopa-oxidase to tyrosine hydroxylase activities was 6.8 for the epidermal homogenate and 6.2 for the CMC eluate.
EPIDERMAL PROTYROSINASE

Fig. 1. Elution profile of frog epidermal protyrosinase from carboxymethyl cellulose. The homogenate of 200 frog epidermis was subjected to preliminary purification as described in the text and the 40 ml solution loaded onto a 2.2 cm × 15.0 cm column of carboxymethyl cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. Washing the column with 260 ml of this buffer at a flow rate of 30 ml/hr removed the unwanted proteins and reduced the absorbance at 280 nm of the eluate to 0.03. The indicated gradient of sodium phosphate buffer, pH 6.8 was applied at the above flow rate and the pure proenzyme eluted as shown.

Carboxymethyl cellulose chromatography. The enzyme was dialyzed against 0.1 M sodium phosphate buffer. After sedimentation for 20 min at 12,000 g, the supernatant fraction was applied to the column at a flow rate of 10 ml cm²/hr. The column (Whatman CM52) was washed with 0.01 M, buffer at the maximum feasible flow rate until the OD₂₈₀ of the effluent reached zero. A linear buffer gradient from 0.01 M to 0.1 M in four column volumes was then applied at a flow rate of 10 ml cm²/hr. The enzyme emerges in a relatively sharp peak centered at 0.03 M (Fig. 1).

PAGE verification of purity. The fraction of highest specific activity appears homogeneous in PAGE at pH 5, 6, and 7. We chose pH 6 for routine analysis of our samples. Figure 2 shows three pairs of pH 6 gels. In these gels, the samples were activated by chymotrypsin before electrophoresis.

Isoelectric points of tyrosinase. The isoelectric points of the proenzyme and active enzyme are not coincident. We estimate the isoelectric point of the proenzyme to be at 9.35 and that of the chymotrypsin-activated material to be 9.20.

Proteolytic activation of tyrosinase. There is no measurable tyrosinase activity in crude frog epidermal extracts or in the purified proenzyme. Only upon proteolysis does enzymic activity appear. We have activated frog protyrosinase with a variety of endopeptidases. Such activation was tested by adding protease to protyrosinase in 0.1 M buffer, incubating at 37°C for 30 min, and then assaying. Chymotrypsin, trypsin, papain and Pronase all activate the enzyme, chymotrypsin being by far the most efficient. Carboxypeptidase A does not activate protyrosinase. Prolonged incubation at 37°C of purified protyrosinase with carboxypeptidase A (1:1 by weight) in 0.025 M Tris–HCl buffer pH 7.5 containing 0.5 M NaCl does not yield activity in the dopa oxidase assay. These conditions are optimal for carboxy-
FIG. 2. Polyacrylamide gel electrophoresis of frog epidermal tyrosinase. Left: 15 \( \mu g \) of the purified proenzyme run on 5\% polyacrylamide gels, pH 6 as described in text. The left gel has been stained for protein with Coomassie Blue 6B and the right gel treated with chymotrypsin and dopa to visualize latent enzymic activity. Center: SDS gel electrophoresis of the proenzyme (left) and chymotrypsin-activated enzyme (right) showing difference in mobility (molecular weight). The 10\% polyacrylamide gels are of the formulation of Weber and Osborn and the sample weight is 45 \( \mu g \). Right: SDS gel electrophoresis of the proenzyme (left) and undialyzed chymotrypsin-activated enzyme (right) showing the low molecular weight peptide which appears on activation. The 15\% polyacrylamide gels have been over-loaded with 150 \( \mu g \) protein to show this peptide which is stained only with difficulty, and the minor bands therefore constitute trace impurities made visible by this overloading. A parallel gel on which was run the amount of chymotrypsin present in the activated enzyme sample did not give visible protein bands. The main peptide has very low mobility on these dense gels and appears as the intense band at the top of the photograph.

The kinetics of the chymotryptic activation suggest rapid proteolysis of some particular peptide bond followed by much slower nonspecific digestion of the molecule. This conclusion is borne out by careful proteolysis of protyrosinase using 2\% CT by weight. SDS gel electrophoresis reveals in the activated enzyme solutions a single small peptide which does not correspond to any trace contaminant of the proenzyme. We estimate the molecular weight of this new peptide to be 5700 ± 700. Figure 2 shows SDS gels of the proenzyme, the active enzyme, and the new peptide that appears following proteolysis.

Early in our studies we considered the possibility that tyrosinase, as extracted from the frog skin, was inactivated by a noncovalently bound inhibitor, the binding
effect of which was destroyed by proteolysis. Consequently, we spent considerable effort attempting to activate the enzyme by removing this hypothetical inhibitor. Prolonged dialysis of the enzyme produced no activation, nor did dialysis against anisate, the most effective competitive inhibitor of dopa oxidation. Urea and SDS were similarly ineffective in evoking activity.

Kinetic results. The pH dependence of dopa oxidase activity was investigated from pH 3 to 11 in a 0.05 M buffers of citric acid, phosphoric acid, glycine, and glycylglycine. Maximum activity occurred at approximately pH 7.0.

Figure 3 shows Lineweaver–Burke Plots of both the tyrosine hydroxylase and dopa oxidase activities of the R. pipiens tyrosinase. It is clear that the enzyme has similar $K_m$ values for both L-tyrosine and L-dopa. The $V$ for both substrates are identical.

Copper-complexing agents also inhibit the enzyme but do not compete with dopa. Diethyldithiocarbamate and cyanide effect 50% inhibition at concentrations of 2.5 and $1 \times 10^{-5}$ M, respectively. Copper can be removed from the proenzyme or active enzyme by dialysis against 10 mM cyanide or diethyldithiocarbamate. Following removal of the complexing agent, prolonged incubation with 1 mM Cu$^{2+}$ restores up to 40% of the activity of the original protein to the apoenzyme, or to the chymotrypsin-treated apoprotein. Alternatively, the copper may be replaced in the apoprotein before proteolysis with the same result. Other divalent cations used at 1 mM concentration in unsuccessful attempts to restore activity to the apoprotein were: Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Fe$^{2+}$.

The enzyme is remarkably stable in SDS. At the highest SDS concentrations used (0.2%), the activity of the enzyme is indistinguishable from that observed when no SDS is present.

Structural characterization of enzyme. The molecular weights of both the active and proenzyme were estimated by gel filtration to be 200,000 ± 12,000. This jibes with a sedimentation coefficient of 10.0 s for both proteins obtained by sucrose gradient centrifugation.

![Lineweaver-Burke plots for dopa-oxidase and tyrosine hydroxylase activities of frog epidermal tyrosinase. Both the Michaelis constant and the turnover number appear similar for both substrates.](image-url)
Both the proenzyme and the active enzyme are apparently tetramers composed of single polypeptide chains, the molecular weights of which are 54,100 ± 700 and 50,000 ± 700, respectively. Figure 4 shows the mobility difference in SDS gel electrophoresis between the two polypeptides. We conclude that the enzyme is a tetramer. The copper content of the proenzyme is 4.8 atoms/200,000 MW, which corresponds to an equivalent weight of 50,000 g per mole of copper. This supports our conclusion that the 54,000 MW protein observed in SDS electrophoresis represents the only polypeptide of the functional subunit.

The amino acid analysis of the proenzyme is shown in Table 2. No correction has been made for loss of cysteine, but it is clear that this amino acid is present in the protein in substantial quantities. The high content of basic residues, when compared with the total of asx and glx, reflects the high isoelectric point observed for the protein.

One interesting point of comparison between the active tyrosinase and the proenzyme is the thermostability of the two proteins. The active enzyme has a thermal inactivation half-time of 120 min at 60°C; half the latent activity of the proenzyme is lost in only 20 min at this temperature. We conclude that proteolysis permits the active enzyme to assume a more compact, and hence a more thermostable structure than that available to the proenzyme.

Role of peroxidase. The crude extract of epidermis contains tyrosinase, dopa oxidase, and catalase activities. The peak tubes from CMC contained tyrosinase and dopa oxidase activities in the same ratio as in the crude extract. The purified preparation had no catalase activity but did catalyse the oxidation of guaiacol in the presence of H2O2. The observation that guaiacol was also oxidized in the absence of H2O2 and in the presence of catalase led us to conclude that guaiacol was another substrate for a relatively nonspecific tyrosinase.

In order to rule out any contribution to dopa or tyrosine oxidation by H2O2, these activities were monitored in the presence of catalase. Excess amounts of

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** SDS-PAGE polypeptide molecular weight determinations for frog epidermal protyrosinase and the chymotrypsin-activated enzyme. Both the technique and the selection of molecular weight standards is that of Weber and Osborn (9). The arrows indicate the mobilities corresponding to the two tyrosinase proteins and imply polypeptide molecular weights of 54,100 and 50,000 for the proenzyme and the active enzyme, respectively.
TABLE 2  
AMINO ACID COMPOSITION OF FROG EPIDERMAL PROTYROSINASE

| Amino acid    | Grams per 100 g protein | Moles per 200,000 MW |
|---------------|-------------------------|----------------------|
| Aspartic acid | 12.66                   | 190.2                |
| Glutamic acid | 9.52                    | 129.4                |
| Glycine       | 4.28                    | 113.8                |
| Alanine       | 3.35                    | 75.3                 |
| Valine        | 4.17                    | 71.2                 |
| Leucine       | 8.49                    | 129.4                |
| Isoleucine    | 4.64                    | 70.8                 |
| Proline       | 5.21                    | 90.5                 |
| Serine        | 4.05                    | 77.2                 |
| Threonine     | 4.45                    | 74.8                 |
| Half-cystine  | (1.43)                  | (23.7)               |
| Methionine    | 2.77                    | 37.1                 |
| Phenylalanine | 8.03                    | 97.3                 |
| Tyrosine      | 8.52                    | 94.1                 |
| Tryptophan b  | 3.45                    | 38.8                 |
| Histidine     | 5.40                    | 69.6                 |
| Lysine        | 10.97                   | 150.0                |
| Arginine      | 13.77                   | 158.1                |

The results are averaged from four analyses of a single representative preparation.

*Cystine is not recovered quantitatively after the hydrolysis.

*b Tryptophan analyses were performed spectrophotometrically (20).

catalase did not influence the oxidation of tyrosine as measured by the Pomerantz assay (5) or the oxidation of dopa as measured by the Fling et al. assay (6). Highly purified tyrosinase from hamster melanoma, a gift from Seymour Pomerantz, was similarly unaffected in its ability to catalyze the oxidation of tyrosine by the addition of catalase. The lack of effect of catalase on tyrosine oxidation is shown in Fig. 5. Purified frog epidermal tyrosinase eluted from a carboxymethyl cellulose column was activated with trypsin and added to tyrosine at room temperature. The oxidation of tyrosine was unaffected by the addition of catalase.

DISCUSSION

We have shown that frog epidermal protyrosinase, because of its high isoelectric point, can be isolated in high purity by a simple high yield procedure.

Of great interest to us is the phenomenon of proteolytic activation. This is an example of posttranslational control whereby the frog's capacity to synthesize melanin can be regulated by proteolytic enzyme(s). Such a control mechanism for tyrosinase activation in higher organisms would be of considerable importance. The best characterized mammalian tyrosinase from mouse melanoma is fully active in crude extracts (13). It is possible, nonetheless, that tyrosinase is synthesized as a zymogen and that disruption of the tyrosinase-bearing tissue releases proteases which activate the proenzyme during its isolation.

The proteolytic activation of frog tyrosinase was originally observed by Purvis who found that trypsin permitted the extraction of tyrosinase from the skin and assumed that trypsin solubilized the enzyme (14). In 1971, Lee and Lee reported that trypsin-activated tyrosinase in the supernatant fraction obtained after homogenization of frog skin (15). In 1970, we reported evidence that the frog epi-
Fig. 5. Effect of catalase on tyrosine oxidation. The additions to each tube are indicated below.

| Tube 1 | Tube 2 | Tube 3 |
|--------|--------|--------|
| Tyrosine | +      | +      | +      |
| Protyrosinase | +      | +      | +      |
| Trypsin | 0      | +      | +      |
| Catalase | 0      | 0      | +      |

The concentrations of the reagents were as follows: tyrosine $4 \times 10^{-3} \text{ M}$, trypsin 100 $\mu$g per tube of Sigma twice crystallized trypsin from beef pancreas, catalase 752 units per tube of Worthington (CTR) twice crystallized from beef liver 50,500 units/mg PO$_4$ buffer pH 7, 0.04 M. 22 $\mu$g of frog epidermal protyrosinase purified by CMC chromatography was added to each. The total reaction volume was 0.5 ml. The tubes were incubated at 25°C and photographed 1 hr after mixing. Tube three contained 2 $\mu$moles of tyrosine and enough catalase to oxidize 752 $\mu$moles of H$_2$O$_2$ per minute. This excess catalase did not influence the rate of tyrosine oxidation.

dermal tyrosinase existed as a protyrosinase (4). Efforts to demonstrate activation of human and guinea pig tyrosinase by proteolytic enzymes have been unsuccessful. Pretreatment of human or guinea pig epidermis with diisopropylfluorophosphate before incubation with dopa or tyrosine fails to inhibit the tyrosinase reaction (unpublished observations, Jegasothy and McGuire). There is recent evidence for increased activity T$_1$ tyrosinase obtained from mouse hair bulb in the presence of trypsin (16).

Szabo was the first to show that melanocytes from white human epidermis form pigment granules in vitro when incubated in tyrosine. He concluded that the theory that tyrosinase was inhibited was incorrect. It may be important in the analysis of his observations to recall that he separated epidermis from dermis with trypsin before the incubation with either dopa or tyrosine (17).

The unitary concept that a single enzyme is responsible for catalyzing the oxidation of both tyrosine and dopa has been challenged by Okun et al. (18), who claim to have demonstrated peroxidase in melanosomes and argue that this peroxi-
dase is responsible for the tyrosine–dopa conversion. Dopa, they feel, is then oxidized in the conventional pathway. Recently Okun et al. have stated that mammalian tyrosinase is only a dopa oxidase with no ability to oxidize tyrosine (19). Our observations with frog tyrosinase indicate that:

Purified frog tyrosinase and purified hamster-melanoma tyrosinase catalyze the oxidation of tyrosine in the presence of excess catalase. Frog tyrosinase oxidizes guaicol in the presence of catalase without added H₂O₂ which suggests that frog tyrosinase is sufficiently nonspecific in its substrate requirements to oxidize guaicol nonperoxidatically.

Our experiments do not establish tyrosinase as the exclusive catalyst for tyrosine and dopa oxidation. They do, however, show that both frog and hamster tyrosinase can catalyze the oxidation of both substrates. It is possible that the nonspecificity of tyrosinase for other substrates such as guaicol has allowed misinterpretation of the role of peroxidase.

SUMMARY

Tyrosinase (o-diphenol:O₂-oxidoreductase, EC 1.10.3.1) is present in the epidermis of R. pipiens as an inactive proenzyme which can be activated by a variety of endopeptidases, the most effective being chymotrypsin. This proenzyme has been obtained in a homogeneous state as judged by polyacrylamide gel electrophoresis.

Protyrosinase and tyrosinase have molecular weights of 200,000 ± 12,000. SDS gel electrophoresis reveals molecular weights of 54,100 ± 700 for protyrosinase and 50,000 ± 700 for tyrosinase. A peptide of molecular weight 5700 ± 1100 appears on chymotryptic activation of the protyrosinase. Copper content of 4.8 atoms per molecule of protyrosinase suggests that protyrosinase is a tetramer with a subunit of approximately 50,000 MW which contains a single atom of copper.

The Kₘ values for both tyrosine and dopa are 5 × 10⁻³ M. The amino acid composition of protyrosinase is consistent with isoelectric points of 9.35 and 9.25 for protyrosinase and tyrosinase. The oxidation of dopa or tyrosine is unaffected by the presence of catalase. This observation renders unlikely a role for peroxidase in the oxidation of tyrosine by frog tyrosinase.

The existence of this posttranslational control of tyrosinase activity offers a potentially important site for regulation of melanin production.

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