TYROSINASE INHIBITION BY ITS APOENZYME AND THE TRANSFORMATION OF A PROTEIN INHIBITOR INTO TYROSINASE*

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Previously we have reported on the presence of a protein, isolated from mushrooms, which inhibited the enzymic activity of tyrosinase (Karkhanis and Frieden, 1961). This protein inhibitor of tyrosinase (PIT) can also cause a lag when the enzyme activity is measured using tyrosine as substrate. Further studies on the mechanism of action of PIT have shown that it can serve as a tyrosinase precursor, being converted to fully active tyrosinase upon an appropriate addition of cupric ion. Apoenzyme prepared from purified tyrosinase also inhibits tyrosinase activity.

EXPERIMENTAL

Preliminary data on the isolation of PIT and tyrosinase have been described earlier (Frieden and Ottesen, 1959; Karkhanis and Frieden, 1961). PIT gives the typical protein tests and its copper content is 0.06%. In the ultracentrifuge PIT is over 95% homogeneous with an S_{20} of 8.2, when extrapolated to zero concentration.

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The spectrophotometric method of Fox and Burnett (Fox and Burnett, 1958) was adapted for determining tyrosinase activity as described earlier (Karkhanis and Frieden, 1961). The 3.0 ml. reaction mixture contained 40 units of tyrosinase, 6.0×10^{-4} M tyrosine, and 0.022 M phosphate buffer, pH 7.0. Incubation experiments with Cu^{++} were as follows: To 2 mg. of PIT, Cu^{++} ions were added to make the total Cu concentration 0.20% or 0.40%, including the amount present in PIT. The final volume was brought to 1.0 ml. in 0.022 M phosphate buffer, pH 7.0. 25 µl of this solution were tested for PIT activity. After the complete loss of PIT activity was noted, the mixture was tested for tyrosinase activity. Lerner's method (1950) for the preparation of apotyrosinase from tyrosinase was used. For the preparation of denatured tyrosinase, tyrosinase was kept at 70°, for five minutes and immediately cooled to 0°.

RESULTS AND DISCUSSION

When Cu^{++} was incubated with PIT at 3°, a steady loss in inhibitory activity was observed (Fig. 1A). After six hours all PIT activity was lost. At this point, the incubation mixture was tested for tyrosinase activity. Enzymic activity was first detected at the seventh hour after which the activity increased rapidly and reached a maximum after sixteen hours (Fig. 1B). It is possible that between 6-7 hours, when neither inhibitory nor enzymic response was detectable, equivalent amounts of enzyme and inhibitor are present. This point may also represent an intermediate state in the conversion of PIT into a tyrosinase. Although the kinetics of deinhibition with a total of 0.40% Cu and 0.20% Cu was the same, a difference was noted in their transformation into an enzyme. Excess Cu^{++} aids in the conversion to a highly active tyrosinase (X).

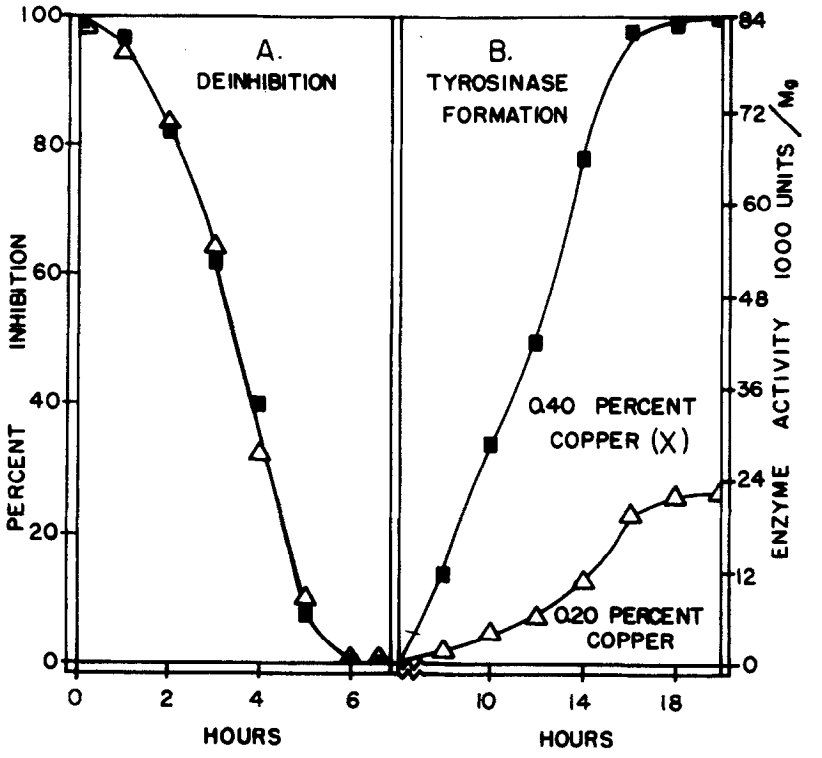

Fig. 1A. Deinhibition of the protein inhibitor of tyrosinase (PIT) with two Cu^{++} concentrations. 50 μg. of Cu^{++}-treated PIT (pH 7.0) which inhibited tyrosinase 100% at zero time, were tested for PIT activity with tyrosinase. The percent inhibition was plotted against the hours of incubation with Cu^{++}. See experimental for other details.

Fig. 1B. Development of tyrosinase activity from PIT on continued incubation with Cu^{++}. These experiments are continuations of the experiments in Fig. 1A. 25 λ, or less, of this mixture were tested for tyrosinase activity in the usual way.

The transformed enzyme (X) prepared by incubation with 0.40% copper, after dialysis, had the following additional properties. The specific activity was 8100 units/ml. per optical density unit at 280 mu

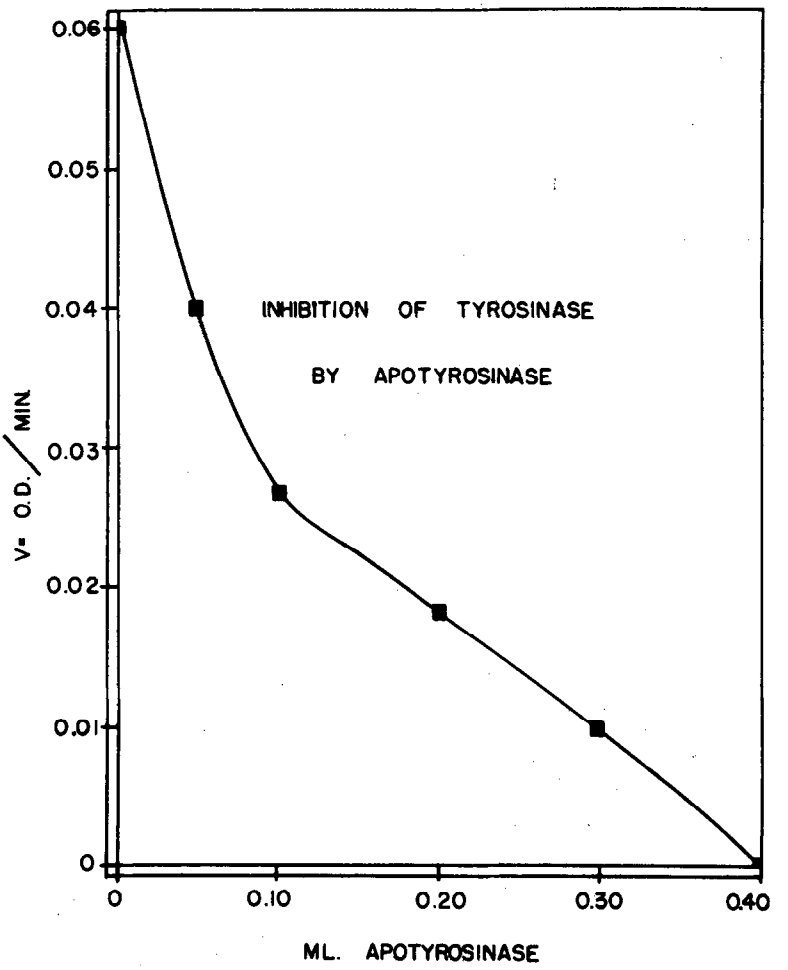

Fig. 2. Inhibition of tyrosinase by apotyrosinase. Apotyrosinase was prepared from 3100 units of tyrosinase after incubation with 0.5 M KCN at 3° overnight, precipitation with saturated $(NH_4)_2SO_4$, standing for 24 hours and extensive dialysis for 48 hours with many changes. From the final total volume of 2.1 ml., the indicated volume of apotyrosinase was tested for its effect in a typical tyrosinase test system.

compared to a specific activity of 10,000 for purified tyrosinase. It contained 0.22% Cu, compared to 0.20% Cu reported for purified tyrosinase (Kertesz and Zito, 1957; Yasunobu, et al., 1957). The Michaelis constant,

Km, was 4×10^{-5} M compared to 5×10^{-5} M for purified tyrosinase. L-Tyrosine, dihydroxy-L-phenylalanine, catechol and L-thyronine were substrated for this preparation; and neocuproine, cyanide, thyroxin and PIT inhibited the activity of (X) to the same extent. The one difference noted was that (X) was less stable than tyrosinase prepared directly from mushrooms.

The activation of PIT to tyrosinase by Cu^{++} is analogous to the reactivation of tyrosinase apoenzyme (Kubowitz, 1937; Lerner, 1950; Kertesz, 1952). The parallel between PIT and apotyrosinase suggested the testing of apotyrosinase as an inhibitor. As shown in Fig. 2, apotyrosinase is also an effective tyrosinase inhibitor. The identity or non-identity of PIT and apotyrosinase is presently under investigation. When tyrosinase is incubated with thyroxin and certain analogs, an inhibitory protein devoid of Cu ion is also produced (Karkhanis and Frieden, unpublished data). Other proteins--heat denatured tyrosinase, ceruloplasmin, lactoglobulin, bovine plasma albumin--did not inhibit tyrosinase.

While it is possible that PIT may be an artifact produced in the isolation process, the existence of naturally occurring protein inhibitors of enzymes is well known. Thus a mechanism of biochemical regulation is conceivable which might include the existence of a series of apoenzymes which can modify the activity of the parent enzyme by direct inhibition. For a metalloenzyme, the inhibition may be due to an intense competition between the apoenzyme and the holoenzyme at the metal ion site. Evidence for a possible inhibitory function of other apoenzymes is now being sought in this laboratory.

REFERENCES

Fox, A.S. and Burnett, J.B., Proc. Soc. Expt. Biol. Med., 98, 110 (1958).

Frieden, E. and Ottesen, M., Biochim. Et Biophys. Acta, 34, 248 (1959).

Karkhanis, Y. and Frieden, E., J. Biol. Chem., 236, PC1 (1961).

Kertesz, D., Biochim. Et Biophys. Acta, 9, 170 (1952).

Kertesz, D. and Zito, R., Nature (London), 179, 1017 (1957).

Kubowitz, F., Biochem. Z., 292, 221 (1937).

Lerner, A. B., Fitzpatrik, T. B., Calkins, E. and Summerson, W. H., J. Biol. Chem., 187, 791 (1950).

Yasunobu, K., Thomson, L. C. G. and Mason, H. S., Fed. Proc., 16, 1176 (1957).