Phytochemistry, Vol. 25, No. 6, pp. 1281–1283, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

FERROXIDASE ACTIVITY OF MUSHROOM TYROSINASE

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(*Revised received* 29 *October* 1985)

Key Word Index—*Agaricus bisporus*; Agaricaceae; mushroom; tyrosinase; ferrous oxidation.

Abstract—Mushroom tyrosinase catalyses the oxidation of Fe(II) to Fe(III). Both the newly-discovered ferroxidase and the well-characterized diphenol oxidase activities of tyrosinase exhibit inhibition by cyanide and both activities co-purify during two preparation steps. The characteristics of tyrosinase-catalysed Fe(II) oxidation are compared with those of other ferroxidases.

INTRODUCTION

Monophenol-*o*-monoxygenase (tyrosinase, EC 1.14.18.1) is a copper-containing enzyme which catalyses the *o*-hydroxylation of phenols and the continued oxidation of the product, *o*-diphenol to *o*-quinones [1, 2]:

$$\text{monophenol} + O_2 \rightarrow \text{catechol} + H_2O$$

$$2\ \text{catechol} + O_2 \rightarrow 2\ o\text{-quinone} + 2H_2O.$$

The enzyme system has been isolated from plants, bacteria and mammals, where it is most likely involved in the biosynthesis of melanins and other pigments. Mushroom tyrosinase, the most thoroughly characterized diphenol oxygenase, is tetrameric and contains four copper atoms which, in the native, oxidized form, are present in antiferromagnetically coupled pairs [3]. The substrate specificity of tyrosinase (mushroom and *Neurospora*) is quite broad and includes many substituted monophenols and *o*-dihydroxyphenyl compounds [4, 5]. One of the best substrates is 3,4-dihydroxyphenylalanine, which has led to the development of a convenient spectrophotometric assay [6, 7]. Pseudoperoxidase activity of mushroom tyrosinase can be demonstrated when ethyl hydroperoxide is included during the enzyme-catalysed oxidation of 4-*t*-butylcatechol under anaerobic conditions [8].

Other copper proteins with much more diverse specificity than tyrosinase have been characterized. For example, ceruloplasmin, the blue copper glycoprotein of mammalian blood, participates in the oxidation of a variety of polyphenols, polyamines and many natural compounds such as ascorbate, adrenaline and 5-hydroxyindoles [9]. Curzon and O'Reilly have demonstrated that Fe(II) enhances the ceruloplasmin-catalysed oxidation of *p*-phenylenediamine [10]. It has since been discovered that Fe(II) is the most active ceruloplasmin substrate, and Frieden has suggested that ceruloplasmin functions as a ferroxidase in the formation of $Fe(III)_2$-transferrin, a transport form of iron in mammals [11]. We have recently reported the apparent ferroxidase activity of mushroom tyrosinase [12]. Here we present our studies on the characterization of the newly-discovered activity.

RESULTS

Tyrosinase, when incubated with ferrous ammonium sulphate and transferrin, leads to the formation of diferric transferrin. The rate of the reaction, as determined by the appearance of diferric transferrin at 460 nm, is a linear function of the level of tyrosinase present (Fig. 1). The influence of initial Fe(II) concentration on the rate of iron oxidation is shown in Fig. 2. At iron concentrations above 500 μM, the auto-oxidation rate becomes greater than 40% of the observed rate, hence rate measurements at higher iron concentrations have limited value. K_m and V_{max} are estimated to be 125 μM and 1.1 μM/min, respectively. All three components (tyrosinase, apotransferrin and ferrous ammonium sulphate) must be present for a measurable rate of diferric transferrin formation. In addition, no reaction occurs under anaerobic conditions.

The enzyme-catalysed oxidation of Fe(II) is inhibited by cyanide. A concentration of 60 μM sodium cyanide causes a 50% inhibition of diferric transferrin formation. Kinetic analysis demonstrated that the inhibition was competitive. Prior heating of tyrosinase to 90° for 10 min leads to 85% inhibition of iron and dihydroxyphenylalanine oxidation.

It was not possible to determine the pH optimum of the ferroxidase activity. As the reaction medium approached pH 7, iron auto-oxidation became the dominant reaction.

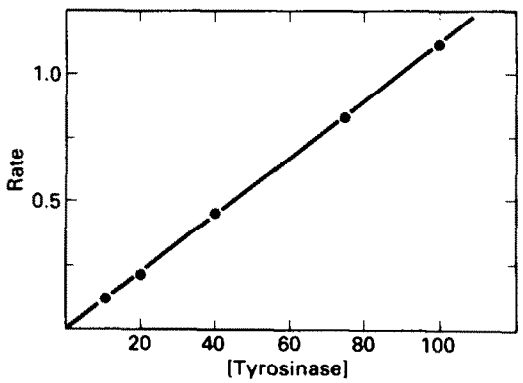

Fig. 1. Tyrosinase catalysis of Fe(II) oxidation. Concentrations were: HEPES buffer (0.2 M), transferrin (150 μM) and ferrous ammonium sulphate (275 μM). Rate units are μM diferric transferrin produced per min. Units of purified tyrosinase are μg/assay.

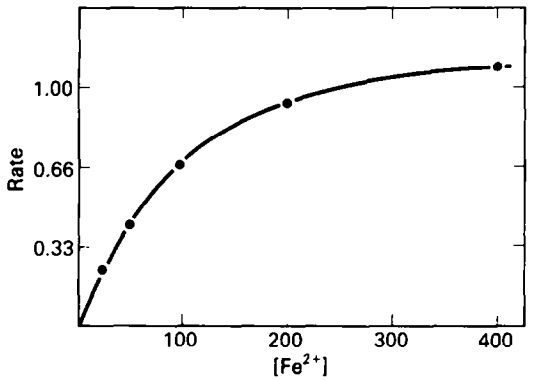

Fig. 2. Initial rate of iron oxidation as a function of Fe(II) concentration. Concentrations were: HEPES buffer (0.2 M), transferrin (150 μM) and purified tyrosinase (0.1 mg). Rate units are μM diferric transferrin produced per min. Iron concentration units are μM.

The pH used in the reported experiments, 6.5, resulted in the greatest measurable enzyme catalysed rate.

The two activities of tyrosinase (ferroxidase and dihydroxyphenylalanine oxidation) were measured during enzyme purification. The data in Table 1 show that there is a constant ratio of the two activities after each purification step.

DISCUSSION

The results, as outlined here, strongly support our initial observation that tyrosinase is able to catalyse the oxidation of Fe(II). To review the results: (1) the initial rate of Fe(II) oxidation is linear with the addition of tyrosinase; (2) the reaction rate dependence on Fe(II) is typical of substrate saturation; (3) the ferroxidase and diphenol oxidase activities are diminished by heat treatment; (4) the two activities co-purify, indicating that the same protein catalyses both dihydroxyphenylalanine and Fe(II) oxidation. Both ferroxidase and catecholase activity are inhibited by sodium cyanide. However, inhibition of catechol oxidase activity is a non-competitive process with respect to the catechol [5], whereas inhibition of Fe(II) oxidation is competitive. Therefore, we believe the two types of substrate, phenol/catechol and Fe(II), bind at different active sites.

It is instructive to compare the ferroxidase activity of tyrosinase with other known copper ferroxidases. Probably the best characterized ferroxidase is the blue copper protein, ceruloplasmin, with M_r of 132 000 and six or seven copper atoms. Over 50 organic substrates have been listed for ceruloplasmin [9]. The ceruloplasmin-catalysed oxidation of Fe(II) has a molecular activity of 550/min and a K_m of 50 μM.

Topham and Frieden have reported the isolation and characterization of a non-ceruloplasmin serum protein, ferroxidase II [13]. This protein is not blue, but does contain copper and requires a phospholipid component and the copper for activity [14]. In contrast to ceruloplasmin, ferroxidase II appears to be specific for Fe(II) and does not catalyse the oxidation of *p*-phenylenediamine or many other aromatic compounds. Although it is difficult to calculate the molecular activity from literature data, it is estimated to be 100–200/min [14].

Table 1. Purification of mushroom tyrosinase

	Activity		
	Diphenol oxidase	Ferroxidase	Ratio
Crude	0.26	0.008	33
Sephadex G-25	0.40	0.013	31
DEAE cellulose	5.1	0.17	31

The columns were monitored for protein elution at 280 nm. Each fraction was assayed for diphenol oxidase activity and ferroxidase activity as described in the Experimental. The activity units for diphenol oxidase activity are ΔA_{475}/min/0.1 mg of protein with 3,4-dihyroxyphenylalanine as substrate. Ferroxidase activity units are ΔA_{460}/min/0.1 mg of protein with ferrous ion as substrate.

A non-copper protein which has recently been characterized as a ferroxidase is xanthine oxidase [15]. The molecular activity is 500 000/min and the K_m is reported to be 46–49 μM. There is evidence to support a role for xanthine oxidase in mucosal processing of iron [16].

As a ferroxidase, tyrosinase has a K_m of 125 μM and a molecular activity of *ca* 150/min. These numbers, compared to other ferroxidases, do not strongly support a physiological role for this activity; however, it may have a significant function in mushrooms or other plants where tyrosinase is present in relatively high concentrations [17]. It should be recognized that the metabolism of iron in plants is not well understood. Ferrous iron is apparently the form taken up by the roots of plants [18], but Fe(III) is formed and stored in phytoferritin by a process not yet defined [19]. Tyrosinase is, to our knowledge, the first plant enzyme to be characterized as a ferroxidase.

It is of interest to consider the types of copper atoms present in those proteins exhibiting ferroxidase activity. Only three copper oxidases (ceruloplasmin, ferroxidase II and tyrosinase) have been extensively tested for ferroxidase activity. Two (ceruloplasmin and tyrosinase) are known to have coupled binuclear copper active sites [3]. The state of copper in ferroxidase II is unknown. It is possible that other proteins with binuclear copper sites (hemocyanin, laccase, ascorbic acid oxidase) have ferroxidase activity.

EXPERIMENTAL

Mushroom tyrosinase, apotransferrin (human, 98%) and ferrous ammonium sulphate were obtained from Sigma. The ferroxidase activity was measured in HEPES buffer, pH 6.5, by a procedure similar to that used for ceruloplasmin [20]. The rate of iron oxidation was determined by monitoring the formation of diferric transferrin at 460 nm using a Hewlett–Packard 8451A diode array spectrophotometer. All rate measurements were taken at 28°. Initial rates were calculated using $E = 4.56$/mM for diferric transferrin [21]. Rate data were corrected for Fe(II) autooxidation.

Mushroom tyrosinase was purified by gel filtration on Sephadex G-25 and ion-exchange chromatography on DEAE cellulose [22]. Both columns were run at 4°. The Sephadex column (40 × 3 cm) was eluted with HEPES buffer, pH 6.5, and the most active fractions pooled for the data in Table 1. The DEAE cellulose column (20 × 2 cm) was eluted with HEPES buffer, pH 6.5, with stepwise increments of NaCl (0.1, 0.2, 0.3 M).

Tyrosinase elution from the columns was detected by both the 3,4-dihydroxyphenylalanine assay [6, 7] (diphenol oxidase) and the ferroxidase assay. Protein measurements were made according to ref. [23].

Acknowledgements—This research was supported by Grant No. 13781-B3-C, Petroleum Research Fund, administered by the American Chemical Society. The spectrophotometer was a generous gift of the Hewlett–Packard Corporation.

REFERENCES

1. Mason, H. S. (1965) *Annu. Rev. Biochem.* **34**, 595.
2. Ullrich, V. and Duppel, W. (1975) in *The Enzymes* (Boyer, P. D., ed.) 3rd edn, Vol. XII, p. 296. Academic Press, New York.
3. Solomon, E. I. (1983) *Pure Appl. Chem.* **55**, 1069.
4. Sussman, A. S. (1961) *Arch. Biochem. Biophys.* **95**, 407.
5. Duckworth, H. W. and Coleman, J. E. (1970) *J. Biol. Chem.* **245**, 1613.
6. Horowitz, N. H., Fling, M., Horn, G. (1970) in *Methods in Enzymology* (Tabor, H. and Tabor, C., eds) Vol. XVIIA, p. 615. Academic Press, New York.
7. Boyer, R. F. (1977) *J. Chem. Educ.* **54**, 585.
8. Strothkamp, K. G. and Mason, H. S. (1974) *Biochem. Biophys. Res. Commun.* **61**, 827.
9. Laurie, S. H. and Mohammed, E. S. (1980) *Coord. Chem. Rev.* **33**, 279.
10. Curzon, G. and O'Reilly, S. (1960) *Biochem. Biophys. Res. Commun.* **2**, 284.
11. Frieden, E. (1971) in *Bioinorganic Chemistry* (Gould, R. F., ed.) Vol. 100, p. 292. American Chemical Society, Washington, DC.
12. Boyer, R. F. and Schori, B. E. (1983) *Biochem. Biophys. Res. Commun.* **116**, 244.
13. Topham, R. W. and Frieden, E. (1970) *J. Biol. Chem.* **245**, 6698.
14. Lykins, L. F., Akey, C. W., Christian, E. G., Duval, G. E. and Topham, R. W. (1977) *Biochemistry* **16**, 693.
15. Topham, R. W., Woodruff, J. H. and Walker, M. C. (1981) *Biochemistry* **20**, 319.
16. Topham, R. W., Walker, M. C., Calisch, M. P. and Williams, R. W. (1982) *Biochemistry* **21**, 4529.
17. Boiret, M., Marty, A. and Deumi, M. (1985) *Biochem. Educ.* **13**, 82.
18. Brown, J. C. (1978) *Plant Cell Environ.* **1**, 249.
19. Clarkson, D. T. and Hanson, J. B. (1980) *Annu. Rev. Plant Physiol.* **31**, 239.
20. Johnson, D. A., Osaki, S. and Frieden, E. (1967) *Clin. Chem.* **13**, 142.
21. Chidambaram, M. V., Barnes, G. and Frieden, E. (1983) *FEBS Letters* **159**, 137.
22. Nelson, R. M. and Mason, H. S. (1970) in *Methods in Enzymology* (Tabor, H. and Tabor, C. W., eds) Vol. XVIIA, p. 626. Academic Press, New York.
23. Bradford, M. (1976) *Analyt. Biochem.* **72**, 248.