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THE SELECTIVE INHIBITION OF CATECHOL OXIDASES BY SALICYLHYDROXAMIC ACID.

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Key Word Index—Catechol oxidase; diphenol oxidase; laccase; enzyme inhibitors; salicylhydroxamic acid; SHAM.

Abstract—Salicylhydroxamic acid (SHAM) has been shown to be a powerful and selective inhibitor of catechol oxidases but was without effect on laccases. Inhabition was non-competitive at concentrations less than 10 μM

INTRODUCTION

Earlier studies in this laboratory [1] have been concerned with the use of selective inhibitors to differentiate between catechol oxidases, E. C. 1.10.3.2 (*ortho*-diphenol oxidases) and laccases, E.C. 1.10.3.1 (*para*-diphenol oxidases). In this paper we report the results of a study of the effects of SHAM upon catechol oxidases and laccases from different sources. SHAM is usually known as a selective inhibitor of cyanide-resistant respiration in higher plants [2]; however Rich and Bonner [3] also noted briefly that it inhibited mushroom catechol oxidase but did not investigate its effect upon laccase.

RESULTS

The nature of the various diphenol oxidase preparations used in these experiments was investigated first by substrate specificity tests with 4-methylcatechol, quinol and toluquinol (1,4-dihydroxytoluene), followed by tests with cinnamic acid which is a specific inhibitor of catechol oxidase [1]. Toluquinol was used as a test substrate for laccase activity because it usually gave higher rates of O_2 uptake [1]. These tests confirmed our previous findings [1] that enzyme preparations from mushroom and potato possessed catechol oxidase activity whilst those from spruce and *Rhus vernicifera* contained laccase. In the course of these experiments it was also noted that mushroom catechol oxidase exhibited substrate inhibition with 4-methylcatechol at concentrations above 1 mM.

Using the above experimental systems the effect of SHAM was investigated and from the results exemplified in Fig. 1 it may be seen that SHAM was a potent and specific inhibitor of catechol oxidase activity but was without effect on laccase activity. Kinetic analysis using both the Dixon [4] and the Direct Linear plots [5] suggested non-competitive inhibition since the K_m values were little affected but the values for V_{max} were reduced.

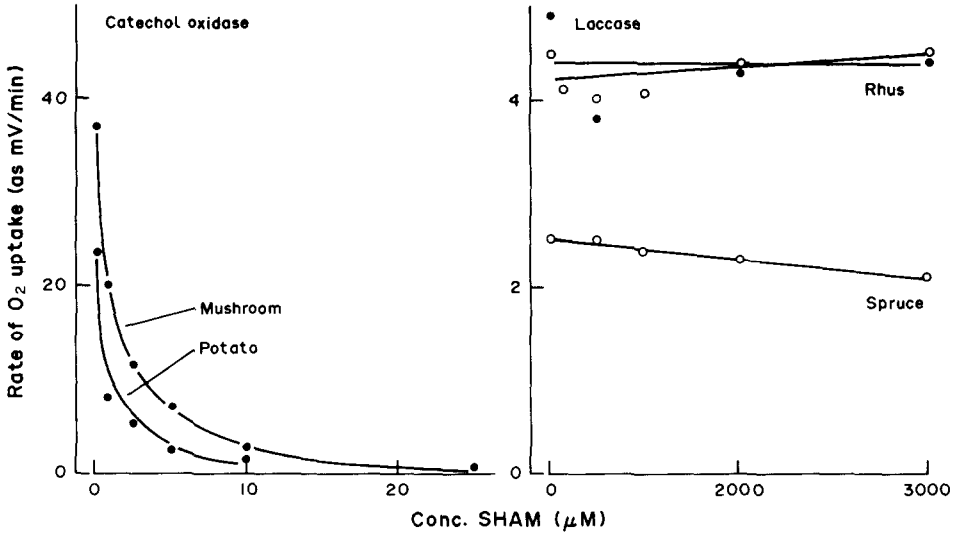

Fig. 1. Effect of SHAM upon diphenol oxidases. 4-Methyl catechol (●) or toluquinol (○) were used as substrates. (Regression equations for the *Rhus* laccase expts were as follows: 4-methylcatchol; $y=4.22-0.045x$, $p=0.401$ toluquinol; $y=4.37-0.026x$, $p=0.777$.)

Catechol oxidase activity was inhibited over 90% by as little as 10 μM SHAM whereas 4 mM SHAM had no effect on laccase activity. The K_i values for SHAM ranged from 0.2 to 2 μM whereas those for cinnamic acid were much higher in the range 0.5–2 mM.

DISCUSSION

The results presented in this paper confirm earlier findings that cinnamic acid was a selective inhibitor of catechol oxidase activity and show that SHAM was an even more potent inhibitor. By contrast neither compound affected laccase activity yet these are both copper-based enzymes capable of oxidizing a wide range of dihydroxyphenols.

The present classification of catechol oxidase and laccase is still rather confusing and this problem has been thoroughly reviewed by Mayer [6, 7]. For example laccases are usually glycoproteins, may contain up to 45% carbohydrate and possess subunit M_rs from 50 000 to 70 000 [6] whereas catechol oxidases are much less variable, with less carbohydrate and subunit weights about 45 000 [8]. Similar differences occur with respect to the role of copper and the nature of the reaction mechanisms [6, 7, 9]; we presume that the selective inhibition by SHAM is due to these differences. Nevertheless it seems surprising that two such similar enzymes behave so differently in the presence of SHAM, but Rich *et al.* [10] have reported that cytochrome *c* oxidase and horseradish peroxidase, two closely-related enzymes with haem-type active sites, were respectively unaffected and potently inhibited by SHAM.

Enzymic browning of fruits and vegetables is usually caused by catechol oxidases and Rich *et al.* [10] reported that 10 μM SHAM effectively prevented the browning of mushrooms and apple slices but there may be doubts about the toxicity of SHAM. However its potent action on catechol oxidase could make it useful for the prevention of enzymic browning during enzyme isolation from plants.

EXPERIMENTAL

Enzyme preparations. Crude extracts of diphenol oxidases were prepared from various plant sources using conventional procedures [1]. Brief details are as follows. Mushroom; basidiocarps of *Agaricus bisporus* (strain AX60) were homogenized in cold Me_2CO, centrifuged, and the pellet resuspended in 0.1 M citrate–Pi buffer, pH 5.5. Potato; (*Solanum tuberosum* var Kaituna, a high-browning variety) was used for the preparation of an Me_2CO powder which was resuspended in pH 5.5 citrate–Pi buffer before use. *Rhus vernicifera*; 1 g of Me_2CO powder of *Rhus* latex (Saito & Co., Tokyo) was suspended in 10 ml pH 5.5 citrate–Pi buffer, centrifuged and the supernatant used as a source of laccase. Spruce (*Picea pungens*); needles frozen in liquid N_2, homogenized, suspended in 0.1 M Pi buffer, pH 7.0 and the supernatant used as a source of laccase.

Enzyme assays. All assays of diphenol oxidase activity were carried out by measuring the initial rate of O_2-uptake using a Rank Bros (Bottisham, UK) O_2-electrode. The reaction cell, held at 30°, contained oxygenated citrate–Pi buffer, pH 5.5 or 7.0, enzyme and the reaction stated by the addition of substrate. K_m and K_i values were estimated using Lineweaver–Burke, Dixon [4] or Direct Linear plots [5].

Chemicals. SHAM was obtained from Sigma whilst 4-methyl catechol and toluquinol were supplied by Fluka.

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REFERENCES

1. Walker, J. R. L. and McCallion, R. F. (1980) *Phytochemistry* **19**, 373.
2. Seidow, J. N. and Berthold, D. A. (1986) *Physiol. Plant.* **66**, 569.
3. Rich, P. R. and Bonner, W. D. (1977) *Plant Physiol.* **59** (suppl), 60.
4. Dixon, M. (1953) *Biochem. J.* **55**, 170.
5. Eisenthal, R. and Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715.
6. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.
7. Mayer, A. M. (1987) *Phytochemistry* **26**, 11.
8. Flurkey, W. H. (1985) *Plant Physiol.* **79**, 564.
9. Holweda, R. A., Wherland, S. and Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng.* **5**, 363.
10. Rich, P. R., Weigand, N. K., Blum, H., Moore, A. L. and Bonner, W. D. (1978) *Biochim. Biophys. Acta* **525**, 325.