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TROPOLONE—A COMPOUND THAT CAN AID IN DIFFERENTIATING BETWEEN TYROSINASE AND PEROXIDASE*

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Abstract—In studies dealing with melanogenesis in mammalian tissues, ultrastructural localization of enzymes, identification of subcellular organelles, differentiation and lignification in plant tissues, it is important to have means to differentiate between tyrosinase and peroxidase activities For a variety of reasons, established criteria used for this purpose are not always reliable We suggest that tropolone can aid in differentiating between tyrosinase and peroxidase activities since (a) it is a very effective inhibitor of tyrosinase, (b) in the presence of hydrogen peroxide it can serve as a substrate for peroxidase, (c) at concentrations that inhibit tyrosinase, it does not inhibit peroxidase activity, and (d) it inhibits tyrosinase activity even in the presence of hydrogen peroxide and peroxidase In a system containing a mixture of tyrosinase and peroxidase, tropolone can differentiate reliably between peroxidase and monohydroxyphenolase or *o*-dihydroxyphenolase activities of tyrosinase Moreover, tropolone can differentiate reliably between peroxidase and tyrosinase activities using slices or crude dialysed extracts of various plant tissues

INTRODUCTION

o-Dihydroxyphenols can be acted upon by tyrosinase as well as by peroxidase and hydrogen peroxide, leading to the formation of *o*-quinones which then undergo rapid non-enzymatic conversion to pigmented polymeric products Tyrosinase can carry out two reactions hydroxylation of monohydroxyphenols to *o*-dihydroxyphenols, and dehydrogenation of *o*-dihydroxyphenols to *o*-quinones, which then polymerize non-enzymatically to form melanins [1] Peroxidase oxidizes various hydrogen donors, AH_2, in the presence of hydrogen peroxide In addition to this peroxidatic reaction, peroxidase, in the absence of hydrogen peroxide, can carry out oxidatic, catalytic and hydroxylation reactions [1] Peroxidase can act on monohydroxyphenols, such as tyrosine, and on *o*-dihydroxyphenols, such as DOPA Hydroxylation of monohydroxyphenols, such as *p*-cresol, tyramine and tyrosine, by peroxidase occurs in the absence of hydrogen peroxide [1] Monohydroxyphenols, such as tyrosine, tyramine and homovanillic acid, can be acted upon by horseradish peroxidase (HRP) in the presence of hydrogen peroxide to form dimers [2] Thus, for example tyrosine, in the presence of hydrogen peroxide and peroxidase, is initially oxidized to dityrosine and is then oxidized to brown polymers [3–6] There is also evidence that DOPA can be converted to dopachrome in the presence of peroxidase and hydrogen peroxide [5]

The fact that tyrosinase, or HRP plus hydrogen peroxide can act on phenols leading to the formation of pigmented products often causes confusion when investigators attempt to determine whether the pigmented products in the tissue were generated by the action of tyrosinase or peroxidase [4, 7–19] This is true in experiments with both animal [4, 7–15] and plant [16–19] tissues

Tropolone (2,4,6-cycloheptatriene-1-one) is an effective copper chelator [20] Goldstein *et al* [21] have shown that tropolone inhibits the copper-containing dopamine-β-hydroxylase We showed in ref [22] that tropolone is an inhibitor of mushroom tyrosinase and that, among various copper chelators tested, it is one of the most potent inhibitors of the enzyme In the course of our work it was also found that tropolone, in the presence of hydrogen peroxide, serves as a substrate for HRP [23] In view of the above, it occurred to us that tropolone can be useful in differentiating between tyrosinase and peroxidase (For terminology used here see ref [22])

RESULTS

We have shown [22] that tropolone can inhibit mushroom tyrosinase possibly because of its ability to chelate copper at the active site of the enzyme Inhibition (50%) occurs at 100, 3 and 0.4×10^{-6} M tropolone when 4-methyl catechol, dopamine and DL-DOPA, respectively, are used as the substrate Moreover, we have shown [23] that tropolone can serve as a substrate for HRP in the presence of hydrogen peroxide The oxidized product formed is yellow, characterized by a peak at 418 nm and suggested to be a tetratropolone [23] Tropolone, in the presence of HRP alone or hydrogen peroxide alone, was not oxidized The oxidation of tropolone to a yellow product occurred only when both HRP and hydrogen

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Table 1 Tropolone as a substrate for HRP in the presence of hydrogen peroxide

HRP (μg)	ΔA at 418 nm/min
0 5	0 24
1 0	0 40
1 5	0 60
2 0	0 74

The reaction mixture included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6 5), 2 2 mM tropolone, 2 2 mM hydrogen peroxide and HRP as indicated The increase in A of 418 nm was linear with time for at least 120 sec Controls of tropolone alone, tropolone plus HRP, tropolone plus hydrogen peroxide or HRP plus hydrogen peroxide had negligible A at 418 nm and gave no increase in A at 418 nm as a function of incubation time

Table 2 Effect of tropolone on HRP activity using guaiacol and hydrogen peroxide as the substrates

No	Tropolone (μM)	Peroxidase activity (ΔA at 470 nm/min)
1	0	0 52
2	80	0 52
3	160	0 52
4	240	0 60
5	320	0 60
6	400	0 60

Peroxidase activity was assayed in a reaction mixture that included, in a total volume of 5 ml, 2 ml guaiacol–hydrogen peroxide mixture [freshly mixed and consisting of 100 ml 0 05 M sodium phosphate buffer (pH 6 5), 10 ml of 1% guaiacol in 50% ethanol and 10 ml of 0 3% hydrogen peroxide], 0 5 μg HRP (added last) and tropolone as indicated

peroxide were present [23] A summary of the data is given in Table 1

The observation that tropolone inhibited mushroom tyrosinase activity and served as a substrate for HRP in the presence of hydrogen peroxide suggested that tropolone could be used as an aid in differentiating between tyrosinase and peroxidase when a mixture of the two is suspected to be present in a homogenate or a tissue In order for this to be effective, it was first necessary to establish that (a) tropolone, at concentrations that inhibit tyrosinase, does not inhibit HRP activity, and (b) tropolone inhibits tyrosinase activity even in the presence of hydrogen peroxide and peroxidase

The data in Table 2 establish that tropolone, in concentrations up to at least 4×10^{-4} M, did not inhibit HRP activity when guaiacol and hydrogen peroxide were the substrates Moreover, at the relatively low concentration of 4×10^{-4} M, tropolone oxidation by HRP plus hydrogen peroxide did not contribute a sufficient increase in A at 470 nm to interfere with the estimation of peroxidase activity, as judged by guaiacol oxidation to a red tetraguaiacol product (A at 470 nm)

The data in Table 3 show that tropolone at a $6\,7 \times 10^{-6}$ M concentration effectively inhibits *o*-dihydroxyphenolase activity of mushroom tyrosinase in the absence, as well as the presence, of hydrogen peroxide

Table 3 Effect of tropolone on *o*-dihydroxyphenolase activity of mushroom tyrosinase in the absence and presence of hydrogen peroxide

Part	Tropolone (μM)	Hydrogen peroxide (mM)	*o*-Dihydroxyphenolase activity (ΔA at 475 nm/min)	(%)
A	0	0	0 52	100
	6 7	0	0	0
	67 0	0	0	0
B	0	0 67	0 56	100
	6 7	0 67	0 03	~5
	67 0	0 67	0 016	~3

The reaction mixture included, in a total volume of 3 ml, 6 7 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6 5), 25 μg mushroom tyrosinase (added last), and hydrogen peroxide and tropolone as indicated

Differentiation between o-dihydroxyphenolase of tyrosinase and peroxidase activities with the use of tropolone

The above results further support our idea that tropolone could be useful in differentiating between tyrosinase and peroxidase A mixture consisting of HRP and mushroom tyrosinase was prepared to test this (HRP–tyrosinase mix) The data in Table 4 illustrate that the HRP–tyrosinase mixture acts on DOPA alone (no hydrogen peroxide), and that this activity is completely inhibited by tropolone, indicating that, in this case, only tyrosinase contributes to the increase in A at 475 nm The oxidation of tropolone by the HRP–tyrosinase mixture occurs only in the presence of hydrogen peroxide (Table 4), indicating the contribution of HRP alone to the increase in A at 475 nm

The data presented in Table 4 show that, in a mixture consisting of peroxidase plus tyrosinase, the activity of each enzyme can be identified with the aid of tropolone The complete inhibition of DOPA oxidation by tropolone in the absence of hydrogen peroxide is a criterion for tyrosinase activity, while the oxidation of tropolone to a yellow product in the presence of hydrogen peroxide is a criterion for peroxidase activity

It should be remembered that tropolone oxidation by HRP in the presence of hydrogen peroxide is best assayed at 418 nm, but the data in Table 4 were obtained at 475 nm for a better comparison with DOPA oxidation by tyrosinase

Table 4 Differentiation between *o*-dihydroxyphenolase activity and peroxidase activity (in a model system) with the use of tropolone

Part	HRP–tyrosinase mix (ml)	DOPA (mM)	Tropolone (mM)	Hydrogen peroxide (mM)	ΔA at 475 nm/min
A	0 1	6 7	—	—	0 09
		6 7	6 7	—	0
		6 7	—	3 3	0 18
		—	6 7	—	0
		—	6 7	3 3	0 35
		6 7	6 7	3 3	0 50
B	0 2	6 7	—	—	0 17
		6 7	6 7	—	0
		6 7	—	3 3	0 34
		—	6 7	—	0
		—	6 7	3 3	0 66
		6 7	6 7	3 3	0 98

Each reaction mixture included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6 5), DL-DOPA, tropolone, hydrogen peroxide as indicated, and either 5 μg HRP plus 5 μg mushroom tyrosinase (part A) or 10 μg HRP plus 5 μg mushroom tyrosinase (part B) (The HRP–tyrosinase mixture was added last)

Differentiation between monohydroxyphenolase activity of tyrosinase and peroxidase activities with the use of tropolone

Monohydroxyphenol hydroxylation by tyrosinase is characterized by an initial lag period This lag period is overcome when a sufficient concentration of *o*-dihydroxyphenol is formed non-enzymatically in the system to serve as a reductant (AH_2) in the hydroxylation reaction [24, 25] or when reductants, such as ascorbate or hydroxylamine, are added exogenously to the system [25–30] Hydrogen peroxide, at relatively low concentrations, can also shorten the lag period [24, 31–33] In the case of mushroom tyrosinase we found that 6 6 mM hydrogen peroxide shortens the lag period of tyrosine hydroxylation by 50% (data not shown)

Peroxidase, in the presence of hydrogen peroxide can convert tyrosine to dityrosine [3–6] There is no lag period in dityrosine formation, but, rather, it is formed immediately upon addition of peroxidase [3–6]

The possible use of tropolone in differentiating between the contribution of tyrosinase and peroxidase to tyrosine hydroxylation was, therefore, tested Using tyrosine as the substrate, an increase in A at 475 nm, by an interaction with the HRP–tyrosinase mix, was observed in the absence and presence of hydrogen peroxide and in the absence and presence of tropolone and combinations thereof It was realized that in such an experiment an increase in A at 475 nm would reflect both dopachrome formation and tropolone oxidation The data in Fig 1 (line A) show that in an interaction of the HRP–tyrosinase mix with tyrosine alone, a lag period of *ca* 4 min was observed before dopachrome formation (an increase in A at 475 nm) could be detected A lag period of *ca* 60 min was observed when the HRP–tyrosinase mix acted upon both tyrosine and tropolone (0 33 or 1 65 mM tropolone) (Fig 1) in the absence of hydrogen peroxide (data shown up to 8 min only) Furthermore, Fig 1 (line B) illustrates

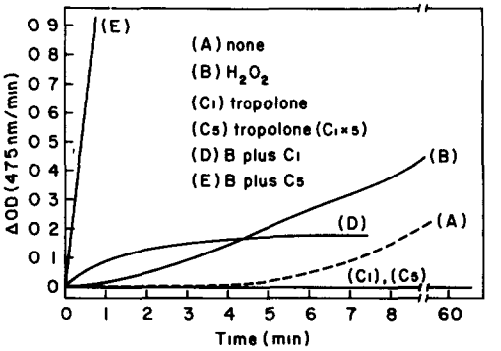

Fig 1 Hydroxylation of tyrosine by the action of a mixture of mushroom tyrosinase and horseradish peroxidase in the absence and presence of tropolone and of hydrogen peroxide Each reaction mixture included, in a total volume of 3 ml, 2 mM L-tyrosine, 47 mM sodium phosphate buffer (pH 6 5) and 0 2 ml enzyme mixture consisting of 10 μg mushroom tyrosinase and 5 μg HRP (HRP–tyrosinase mix) (added last) The following additions were also made A, none, B, 3 3 mM hydrogen peroxide, C_1, 0 33 mM tropolone, C_5, 1 65 mM tropolone, D, 3 3 mM hydrogen peroxide plus 0 33 mM tropolone, E, 3 3 mM hydrogen peroxide plus 1 65 mM tropolone

the ability of 3 3 mM hydrogen peroxide to shorten the lag period of tyrosine hydroxylation to less than 1 min due to a shortening of the lag period of tyrosine hydroxylation by tyrosinase mentioned above [24, 28, 29, 31] Addition of tropolone to a reaction mixture consisting of the HRP–tyrosinase mix, tyrosine and hydrogen peroxide completely abolished the lag period (Fig 1, D, E) and also stimulated the rate of increase of A at 475 nm following the lag period The rate of increase of A at 475 nm was less pronounced in the presence of 0 3 mM tropolone and very

pronounced in the presence of 1 6 mM tropolone (compare Fig 1 D vs E)

The data in Fig 1 illustrate that (a) in the absence of hydrogen peroxide, inhibition of dopachrome formation by incubating tyrosine plus tropolone, compared with incubating tyrosine alone, is a reflection of the contribution of tyrosinase activity in the HRP–tyrosinase mix, and (b) tropolone in the presence of hydrogen peroxide stimulates the increase in A at 475 nm considerably more than does hydrogen peroxide alone This is a reflection of the action of HRP

It can, therefore, be concluded that tropolone can differentiate between peroxidase, and monohydroxy- and *o*-dihydroxyphenolase activity of tyrosinase in a model system containing a mixture of the two enzymes This idea was also tested on several plant tissues (fruits of papaya, banana, avocado and squash, tubers of potatoes, roots of radish and horseradish, and whole edible mushrooms) Dialysed crude extracts were prepared from each tissue (as described in the Experimental) In order to test our idea of the possible use of tropolone in differentiating between peroxidase and tyrosinase activities (see above), thin slices and dialysed crude extracts were also incubated under two sets of conditions

(1) Conditions described in Table 5, namely with the following additions (a) no addition (control), (b) DL-DOPA, (c) tropolone, (d) DL-DOPA and tropolone, (e) hydrogen peroxide, (f) DL-DOPA and hydrogen peroxide, (g) tropolone and hydrogen peroxide, and (h) DL-DOPA, tropolone and hydrogen peroxide Formation of a red-brown colour in the presence of DL-DOPA alone (b) and the inhibition of such colour formation in the presence of DL-DOPA plus tropolone (d) was taken to indicate the presence of tyrosinase activity Formation of a red-brown colour in the presence of DL-DOPA plus hydrogen peroxide (f) but not in the absence of hydrogen peroxide (b) and the observation that tropolone does not inhibit colour formation by the presence of DL-DOPA plus hydrogen peroxide (f) were taken to indicate the presence of peroxidase activity Appearance of air bubbles upon addition of hydrogen peroxide was taken to indicate catalase activity On the basis of the above criteria, the data in Table 5 (for thin slices only) demonstrates that papaya possesses peroxidase but not tyrosinase Radish, horseradish and squash possess peroxidase but not tyrosinase Banana, avocado and potatoes possess peroxidase and tyrosinase, and mushroom possesses tyrosinase but not peroxidase In the case of papaya, banana, avocado, potato and mushroom, air bubbles were formed when hydrogen peroxide was added to the tissue slices, indicating the presence of catalase

(2) Conditions described herein, namely that peroxidase activity was assayed in the presence of guiacol, hydrogen peroxide and sodium phosphate buffer (pH 6 5), and tyrosinase activity was assayed in the presence of DL-DOPA and sodium phosphate buffer (pH 6 5) (as described in detail in the Experimental) Using the dialysed crude extracts as the enzyme source and the above assays it was found (data not shown) that banana, avocado and potato possess peroxidase and tyrosinase activities, papaya possesses peroxidase but not tyrosinase activity, radish, horseradish and squash possess peroxidase but no tyrosinase and mushroom possesses tyrosinase but no peroxidase activity The results obtained under conditions (1) and (2), described above, are clearly the same, thereby establishing that tropolone can indeed

Table 5 Differentiation with the aid of tropolone between *o*-dihydroxyphenolase of tyrosinase activity and peroxidase activity in fresh tissue slices

	Additions			Tissue examined							
Part	DL-DOPA (mM)	Tropolone (mM)	Hydrogen peroxide (mM)	Papaya	Banana	Avocado	Potato	Radish	Horseradish	Squash	Mushroom
A	0	0	0	Orange	Beige-light yellow	Light green	White	White	White	Light green	Beige
B	12	0	0	Orange	Dark red	Conductive vessels brown	Red	White	White	Light green	Red
C	0	12	0	Orange	Beige-light yellow	Light green	White	White	White	Light green	Beige
D	12	12	0	Orange	Pink only in centre	Light green	White	White	White	Light green	Beige
E	0	0	60	Orange	Beige-light yellow	Light green	White	White	White	Light green	Beige
F	12	0	60	Beige	Violet-red	Conductive vessels brown	Red	Brown-red	Red	Pink	Red
G	0	12	60	Brown	Violet-brown	Brown	Brown	Brown-red	Brown	Dark brown	Beige
H	12	12	60	Brown	Violet	Brown	Red-brown	Brown-red	Brown	Brown	Beige

Freshly cut slices of the tissues indicated were placed in Petri dishes (4 cm diameter) and covered with 3 ml of a solution containing DL-DOPA, tropolone and hydrogen peroxide as indicated The colour of the untreated freshly cut tissues was that described in part A

differentiate between peroxidase activity and tyrosinase activity in various plant tissues

DISCUSSION

Investigators studying melanogenesis in mammalian tissues, ultrastructural localization of enzymes, identification of subcellular organelles, and differentiation and lignification in plant tissues, need reliable criteria to distinguish between tyrosinase and peroxidase activities both *in vitro* and *in vivo* The initial step of melanogenesis involves the hydroxylation of tyrosine by the tissue Scientists often disagree as to whether tyrosinase or peroxidase is the enzyme responsible for the hydroxylation of tyrosine to DOPA in melanogenesis [4, 10–15] Thus, for example Patel *et al* [10] concluded that both tyrosinase and peroxidase can convert tyrosine or DOPA to melanin and suggested that these two enzymes are responsible for melanogenesis, while Smith and Swan [5] ruled out the possibility that tyrosine was hydroxylated to DOPA by peroxidase and hydrogen peroxide More recently, Vijayan *et al* [14] made use of phenylthiourea, a specific inhibitor of tyrosinase as a tool to prove that the hydroxylation of tyrosine to DOPA by a tyrosinase preparation of human skin was due to tyrosinase

Problems associated in attempts at differentiating between tyrosinase and peroxidase activities are also encountered in plant research [16–18] Cell differentiation in higher plants is correlated with changes in the behaviour of peroxidase Cytochemical methods are usually employed for the ultrastructural localization of PPO and peroxidase activities in the plant cell [17] Peroxidase is involved in the formation of lignin in plants and it is often suggested that a histochemical localization of peroxidase activity is a very reliable indication of the localization of lignification in tissue [18] Peroxidase, in the presence of hydrogen peroxide, acts on substrates, such as guaiacol and benzidine, while tyrosinase cannot use these In general, substrates acted upon by tyrosinase in the absence of hydrogen peroxide can also be acted upon by peroxidase, in the presence of hydrogen peroxide

Criteria often used in plant studies for the identification of peroxidase and tyrosinase are (a) a positive reaction (pigment formation) in the presence of a peroxidase substrate (i e guaiacol, benzidine) and hydrogen peroxide, but a strong inhibition of the reaction with cyanide, azide or by removal of hydrogen peroxide [18, 19], and (b) a positive reaction (pigment formation) in the presence of DOPA vs a negative reaction by incubation in the presence of DOPA and DETC The above criteria are not always reliable to allow a definite differentiation between tyrosinase and peroxidase activities, regardless of the tissue under study This is due to the fact that inhibitors, such as DETC, inhibit not only tyrosinase but also peroxidase non-selectively Potassium cyanide, azide and phenylhydrazine are known inhibitors of HRP [34, 35], as well as of tyrosinase [36], yet inhibition by these compounds is often suggested as a criterion for peroxidase activity [34]

Peroxidase and tyrosinase are iron- and copper-containing enzymes, respectively The use of chelators specific for either iron or copper, with a concomitant decrease in melanin formation could, theoretically, aid in distinguishing between the two enzymatic activities However, metal chelators are apparently not sufficiently selective to chelate exclusively either copper or iron and, therefore, these chelators actually inhibit, to different extents, both peroxidase and tyrosinase activities

The stimulation by hydrogen peroxide exerted on melanin formation from tyrosine [7, 10], or the decrease in melanin formation due to exogenously added catalase [8], are criteria often used to prove peroxidase rather than tyrosinase activity However, the reliability of the above criteria is questionable in view of the fact that hydrogen peroxide can shorten the lag period of tyrosine hydroxylation by tyrosinase from various sources [24, 31–33] We have recently shown in some detail the relationship between hydrogen peroxide concentration and the shortening of the lag period of tyrosine hydroxylation by avocado PPO [31], we obtained similar results with mushroom tyrosinase (data not shown) Therefore, the stimulation of melanin formation from tyrosine by hydrogen peroxide cannot be used as proof of peroxidase activity Indeed, Hearing [13] pointed out that exogenous hydrogen peroxide can increase melanogenic activity non-specifically, and Patel *et al* [9, 10] questioned the effectiveness of catalase, added exogenously to the tissue, as an inhibitor of peroxidase Moreover, in some tissues some hydrogen peroxide is apparently generated endogenously [37, 38] so that a lack of stimulation by exogenously added hydrogen peroxide might lead to a misleading conclusion

The data presented in this paper establish that tropolone can be useful in differentiating between tyrosinase and peroxidase activities when a mixture of the two enzymes is under study in a model system as well as in tissues from various plant sources Tropolone might be very useful as a means of differentiating between tyrosinase and peroxidase, this being true especially in cases when the increased conversion of tyrosine to pigmented products is under study Under such conditions, the inhibition of tyrosine conversion to pigmented product by tropolone in the absence or presence of hydrogen peroxide would indicate tyrosinase activity, while stimulation of the formation pigmented products by tropolone only in the presence of hydrogen peroxide would indicate peroxidase activity Our suggestion has the advantage that the same compound, namely tropolone, can be of aid in differentiating between tyrosinase and peroxidase activities in either plant or animal tissues, without the use of any additional compound In conclusion, the use of tropolone offers the following Inhibition of the formation of pigmented products (400–475 nm *A* range) from tyrosine or DOPA, in the absence of hydrogen peroxide, by very low concentrations of tropolone reflects tyrosinase activity, while stimulation of the formation of pigmented products from tyrosine or DL-DOPA, in the presence of hydrogen peroxide, by relatively high concentrations of tropolone, reflects peroxidase activity An additional proof of the presence of peroxidase activity is the formation of a yellow product when torpolone is incubated in the presence of hydrogen peroxide but not in its absence

EXPERIMENTAL

Tyrosinase activities Monohydroxyphenolase and *o*-dihydroxyphenolase activities were assayed at 24° in a total reaction mixture of 3 ml that included 2 mM L-tyrosine or 6 7 mM DL-DOPA, respectively, 47 mM NaPi buffer (pH 6 5) and mushroom tyrosinase (added last) as indicated In both cases, the rate of formation of dopachrome as a function of time was

followed at 475 nm in a Varian 635 spectrophotometer equipped with a recorder The lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the *x*-axis, as suggested in ref [39] *o*-Dihydroxyphenolase activity was computed from the initial linear portion of each curve and expressed as *A* at 475 nm/min

Peroxidase activity Assayed at 24° using either tropolone or guaiacol as the substrate (in the presence of H_2O_2), as described in the legends to Tables 1 and 2, respectively

H_2O_2 *concentration* Estimated at 240 nm using an E_m (240 nm) of 43 6/M cm

Preparation of dialysed crude extracts of different plant tissues and assays of enzymes activities Freshly peeled tissue (40 g) was homogenized in an Omni mixer for 5 min with 120 ml NaPi buffer (pH 6 5) The crude homogenate was passed through four layers of cheese-cloth, centrifuged at 6000 *g* for 15 min and the resultant supernatant dialysed overnight against 0 05 M NaPi buffer (pH 6 5) Following dialysis, the supernatant was centrifuged for 10 min at 10000 *g* and used as the enzyme source (referred to as the dialysed crude extract). Thin slices were also prepared from the same freshly peeled tissue from which the extract had been prepared

Enzyme assays on the dialysed crude extracts Peroxidase activity of the dialysed crude extract was assayed in a reaction mixture that included, in a total vol of 3 ml, mix M [freshly mixed and consisting of 100 ml 0 05 M NaPi buffer (pH 6 5), 10 ml 1% guaiacol in 50% EtOH and 10 ml 0 3% H_2O_2] and various aliquots of the dialysed crude extract (added last) The rate of formation of tetraguaiacol was followed at 470 nm and peroxidase activity (ΔA/min) was calculated from the initial curves obtained

Tyrosinase activity of the dialysed crude extract was assayed in a reaction mixture of total vol 3 ml that included 6 7 mM DL-DOPA, 40 mM NaPi buffer (pH 6 5) and various aliquots of the dialysed crude extract (added last) The rate of formation of dopachrome, followed at 475 nm, and tyrosinase activity (ΔA 475 nm/min) was calculated from the initial linear rates of the curves obtained

Materials Mushroom tyrosinase (grade III), horseradish peroxidase (type VI), tropolone, DL-DOPA, tyrosine and guaiacol were obtained from Sigma, H_2O_2 from Merck, all other chemicals were reagent grade

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REFERENCES

1 Whitaker, J R (1972) in *Principles of Enzymology for the Food Sciences* Marcel Dekker, New York
2 Hirsh, H E and Parks, M E (1982) *Analyt Biochem* **122**, 79
3 Gross, A and Sizer, I (1959) *J Biol Chem* **234**, 1611
4 Patel, R P, Okun, M R, Edelstein, L M and Carighia, N (1974) *J Invest Dermatol* **63**, 374
5 Smith, P I and Swan, G A (1976) *Biochem. J* **153**, 403
6 Bayse, G S, Michaels, A. W and Morrison, M (1972) *Biochim. Biophys Acta* **284**, 34
7 Okun, M., Edelstein, L, Or, N, Hamada, G and Donnellan, B (1970) *J Invest Dermatol* **55**, 1
8 Okun, M R, Donnellan, B, Patel, R P and Edelstein, L M (1973) *J Invest Dermatol* **61**, 60
9 Patel, R P, Okun, M R, Yee, W A, Wilgram, G F and Edelstein, L M (1973) *J Invest Dermatol* **61**, 55
10 Patel, R P, Okun, M R, Edelstein, L M and Carighia, N (1974) *Biochem J* **142**, 441
11 Mufson, R A (1975) *Arch Biochem Biophys* **167**, 738
12 Shapiro, H C, Edelstein, L, Patel, R P, Okun, M R, Blackburn, M, Snyder, M, Breman, T and Wilform, G (1979) *J Invest Dermat* **72**, 191
13 Hearing, V (1973) *Arch Biochem. Biophys* **158**, 720
14 Vijayan, E, Husain, I, Ramaiah, A and Madan, N C (1982) *Arch Biochem Biophys* **217**, 738
15 Mondal, M and Banerjee, P K (1981) *Indian J Biochem Biophys* **18**, 380
16 Van Loon, L C (1971) *Phytochemistry* **10**, 503
17 Henry, J (1975) *J Microscop* **22**, 109
18 Helper, P K, Rice, R M and Terranova, W A (1972) *Can J Botany* **50**, 977
19 Menzel, D (1979) *J Histochem Cytochem* **27**, 1003
20 Cook, J W, Gibb, A R, Raphael, R A and Somerville, A R (1951) *J Chem Soc* 503
21 Goldstein, M, Lauber, E and McKereghan, M R (1964) *Biochem Pharmacol* **13**, 1103
22 Kahn, V and Andrawis, A (1985) *Phytochemistry* **24**, 905
23 Kahn, V and Andrawis, A (1985) *Phytochemistry* **24**, 909
24 Jolley, R L, Evans, L H, Makino, N and Mason, H S (1974) *J Biol Chem* **249**, 335
25 Vanneste, W H and Zuberbuhler, A (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayashi, O, ed), p 371 Academic Press, New York
26 Nakamara, T and Sho, S (1964) *J Biochem (Tokyo)* **55**, 510
27 Sato, M (1969) *Phytochemistry* **8**, 353
28 McIntyre, R J and Vaughan, P F T (1975) *Biochem. J* **149**, 447
29 Kahn, V and Pomerantz, S H (1980) *Phytochemistry* **19**, 379
30 Schoot Uiterkamp, A J M, Evans, L H, Jolley, R L and Mason, H S (1976) *Biochim Biophys Acta* **453**, 200
31 Kahn, V (1983) *Phytochemistry* **22**, 2155
32 Vaughan, P F T and McIntyre, R J (1975) *Biochem J* **151**, 759
33 Gutteridge, S and Robb, D (1975) *Eur J Biochem* **54**, 107
34 Herzog, V and Fahimi, H D (1973) *Analyt Biochem* **55**, 554
35 Straus, W (1972) *J Histochem Cytochem* **20**, 949
36 Mayer, A M and Harel, E (1979) *Phytochemistry* **18**, 193
37 Swan, G A and Wright, D (1954) *J Chem Soc* 381
38 Gillet, J R, Watland, D and Kalintsky, G (1954) *Biochim Biophys Acta* **15**, 526
39 Pomerantz, S H and Warner, M C (1967) *J Biol Chem* **242**, 5308