Phytochemistry, 1979, Vol. 18, pp. 25–28. ©Pergamon Press Ltd. Printed in England. 0031-9422/79/0101-0025 $02.00/0

THE MASKING OF PEROXIDASE-CATALYSED OXIDATION OF IAA IN *VIGNA**

T. STONIER, S. STASINOS and K. B. S. MURTHY REDDY†

School of Science and Society, University of Bradford, Bradford, West Yorkshire, BD7 1DP, U.K. and A.P.S. College, Bangalore 560 019, India

(*Revised received* 17 *July* 1978)

Key Word Index—*Vigna sinensis*; Leguminosae; cowpea; auxin protector; IAA-oxidase; peroxidase; phenylpropanoids.

Abstract—Partially purified enzyme preparations of extracts of *Vigna* seedlings exhibited guaiacol-oxidase activity but not IAA-oxidase activity. However, by ageing the enzyme preparations, or by treating them with H_2O_2, it was possible to unmask IAA-oxidase activity. Gel filtration of *Vigna* extracts on Sepharose yielded separate peaks for IAA-oxidase, guaiacol-oxidase and auxin protectors. The appearance of a separate IAA-oxidase peak reflected the overlap of peroxidase and protector; the apparent difference in the migration rate of IAA-oxidase and guaiacol-oxidase activity proved to be an artifact. The data imply that previous reports of differences between peroxidase and IAA oxidase need to be reinvestigated to rule out the possible effect of contamination by endogenous, high MW auxin protectors. A rapid method for removing most of the auxin protectors and thereby unmasking IAA-oxidase activity is described.

INTRODUCTION

Peroxidases (EC 1.11.1.7.) are widely distributed in nature and are associated with numerous catalytic functions (as reviewed in [1]). Among these is the ability to oxidise IAA [2, 3]. Multiple isozymes of peroxidase exist in various plants [4]. There is considerable confusion in the literature over the relationship between peroxidase and IAA-oxidase. This can be summarized by considering 4 hypotheses: (a) peroxidase and IAA-oxidase are different enzymes [5, 6]; (b) there exist several peroxidase isozymes on which only some possess IAA-oxidase activity [7]; (c) peroxidase and IAA-oxidase activities are associated with the same protein molecule but they have different active sites or involved differential allosteric activation [8–12]; (d) peroxidase and IAA-oxidase are identical; IAA is merely another substrate [13, 14].

Various isoperoxidases isolated from tobacco cells invariably showed IAA-oxidase activity [15]. The same was observed with isoperoxidases isolated from pea stems, but there was a marked difference among some of them in their apparent ability to oxidize the various substrates [16]. Isoperoxidase isolated from tobacco cell suspension culture exhibited similar phenomena [17].

The purpose of this paper is to investigate the relationship between peroxidase and IAA-oxidase. Much of the confusion in the literature derives from the 'masking' of the IAA-oxidase activity in plant extracts by the intimate association of the enzyme with high MW auxin protectors (Prs) [18]. The data show that it is possible to obtain separate peaks of IAA-oxidase and guaiacol-oxidase activity following gel filtration and yet still deal with only a single enzyme.

RESULTS AND DISCUSSION

Preliminary experiments and observations

Extracts of *Vigna* hypocotyls invariably showed peroxidase activity. This activity was heat-labile, non-dialysable and precipitated with either acetone or ammonium sulfate. The partially purified enzyme exhibited high peroxidase activity, but no IAA-oxidase activity under the conditions tested. In one experiment, the enzyme preparation was further purified by means of Sephadex column chromatography. The enzyme eluted from the column as a single peak and again exhibited no IAA-oxidase activity in those fractions which were tested. Acrylamide gel electrophoresis showed *ca* 7 bands of isoperoxidases but no IAA-oxidases, although on one occasion two very faint bands of IAA-oxidase did appear when stained by the method of Endo [19].

In summation, partially purified *Vigna* extracts contained enzymes with peroxidase activity but lacked any clearly demonstrable IAA-oxidase activity. There remained, however, the possibility that IAA-oxidase activity was present but masked by endogenous auxin protectors.

Unmasking IAA-oxidase activity in peroxidase preparations

A high MW fraction of *Vigna* extracts was obtained by separation on Sephadex G-200. This preparation exhibited classical peroxidase activity, but lacked IAA-oxidase activity. However, when the enzyme co-factors/IAA mixture was allowed to incubate for long periods, the destruction of IAA could be observed. Furthermore, a rapid destruction of IAA could also be observed upon

*Part XIII in the series "Studies on Auxin Protectors". For Part XII, see ref. [22].

†Present address: Fruit and Vegetable Technology Division, Central Food Technology Research Institute, Mysore 570 013, India.

Table 1. Comparison of IAA-oxidase and peroxidase (guaiacol-oxidase) activity in high MW Sephadex G200 fractions

Sephadex fraction	Peroxidase*	H_2O_2 treatment	IAA-oxidase† IAA not oxidized after incubation period 0.25 hr	0.5 hr	17 hr
1	0.005	–	0.142	0.145	0.130
		+	0.115	0.111	0.115
2	0.182	–	0.148	0.150	0.105
		+	0.095	0.089	0.095
3	0.800	–	0.147	0.131	0.022
		+	0.032	0.022	—
4	0.675	–	0.150	0.145	0.021
		+	0.032	0.022	—
5	0.315	–	0.140	0.138	0.070
		+	0.080	0.072	0.020

*Oxidation of guaiacol: *A* at 540 nm after 0.5 hr incubation.
†Destruction of IAA: Salkowski test, *A* at 540 nm.

the addition of 10 mM of H_2O_2 (summarized in Table 1).

The data (Table 1) indicate that a peak of guaiacol oxidase activity was eluted in fractions 3–4. Whereas guaiacol was rapidly oxidized in 30 min, virtually no IAA was oxidized. However, IAA was oxidized when the mixtures were allowed to incubate for 17 hr. This 'spontaneous' oxidation of IAA correlated with the guaiacol oxidizing ability: only slight IAA oxidizing activity could be observed (even after 17 hr) where only slight guaiacol activity could be observed (e.g. tube 1).

This 'ageing' effect on unmasking IAA oxidase activity could be achieved instantly by the addition of 10 mM H_2O_2. In the presence of H_2O_2, fractions 3–4 could completely oxidize IAA in 30 min (as well as guaiacol). The other fractions also showed IAA oxidase activity corresponding to guaiacol activity, although the data are complicated by the fact that at the high concentration, the H_2O_2 is involved in several reactions which change the kinetics of the IAA-oxidation.

Table 2. Comparison of auxin-protector, guaiacol-oxidase and IAA-oxidase activity in various Sepharose fractions of *Vigna* shoot extract

Sepharose fraction	Auxin protector lag in hr	Peroxidase Guaiacol *A* at 540 nm after 15 min	IAA-oxidase Salkowski *A* at 540 nm after 90 min*
1			0.092
2	0	0.032	
3			0.082
4	0	0.082	
5			0.038
6	0	0.205	
7			0.020
8	0	0.340	
9			0.013‡
10	0	0.490	
11			0.020
12	0.03	0.540†	
13			0.120
14	0.75, 2	0.485	
15			0.117
16	3, 6	0.380	
17			0.125
18	6	0.280	
19			0.113
20	6†	0.195	
21			0.120
22	6	0.125	
23			0.128
24	3, 6	0.082	
25			0.120
26	0.75, 2	0.062	

*Controls lacking enzyme were reading 0.125.
†Peak of activity tested.

Separation of peroxidases and auxin protector activity

The above data are consistent with previous observations on extracts from other plant species which contain significant amounts of auxin protectors. Assaying the Sephadex G-200 fractions in the above experiments did indeed show the presence of an auxin protecting fraction which migrated more slowly than the peroxidase. To improve separation, the extracts were filtered through Sepharose CL-4B in subsequent experiments. Typical results are described in Table 2. A peak of peroxidase activity may be observed at fraction 12, while the Prs peak is around fraction 20. Substantial IAA oxidase was found with a peak of activity in fraction 9, i.e. the other side of the peak from where the Pr activity appears. Thus it is possible not only to separate Pr from peroxidase, but to obtain two distinct peaks of guaiacol and IAA-oxidase activity.

A closer inspection of Table 2 shows that where the peroxidase and Pr activity overlap, no IAA-oxidase activity is observed. The masking of IAA-oxidase can be seen by noting that in front of the peroxidase peak at tube 12 no protector activity is detected, while behind it one can detect no IAA-oxidase activity.

A second indication that the separate IAA-oxidase peak represents a masking effect, rather than a separate enzyme, is the shape of the peaks: protector activity elutes as a symmetrical peak. Peroxidase is slightly asymmetrical, the front being slightly steeper than the back; a 'tailing' effect frequently encountered in this type of procedure. In contrast, the IAA-oxidase activity gradually increases in tubes 1–9, and in parallel with the guaiacol activity. It then drops dramatically from almost maximum activity in tube 11 to zero activity in tube 13.

A third line of evidence which implies that the IAA-oxidase activity is present but masked in those fractions which exhibit guaiacol activity is based on the following: ageing those fractions under oxidative conditions which favour the destruction of the Pr can lead to the appearance of IAA-oxidase. This ageing process may be accomplished by incubating the peroxidase-containing fraction at 30° in the presence of the co-factors needed for the oxidative process (Mn plus DCP). For example, samples from tube 15 (Table 2) were incubated for 22 hr in the presence and absence of oxidising co-factors. Samples incubated without co-factors still exhibited no IAA-oxidase activity. Those incubated with co-factors showed activity after a 1–2 hr lag. The data imply that even the 'aged' fraction still contained enough Pr to produce a lag which would account for the fact that not all tubes 'aged' for 17 hr yielded IAA-oxidase activity.

Finally, if IAA-oxidase is masked by the presence of Pr in the fractions beyond tube 12, then it should be

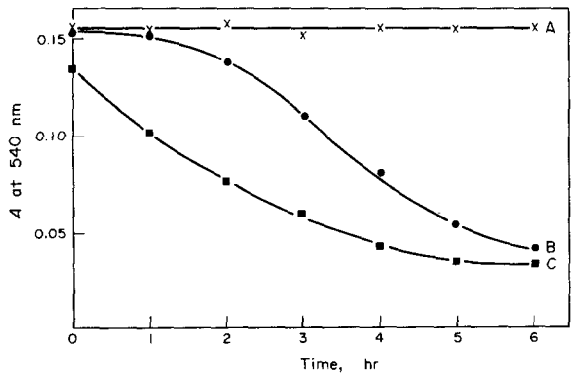

Fig. 1. IAA-oxidase activity of crude *Vigna* extracts before and after 'unmasking'. (A) no treatment; (B) gel filtration only; (C) H_2O_2 followed by gel filtration.

possible to mask the IAA-oxidase in tubes 1–11 by the addition of fractions containing Prs (tubes 14–26). This was observed. For example, adding an equal volume of tube 21 to tube 9 (which showed a peak in IAA-oxidase activity) resulted in a complete loss of IAA-oxidase activity (zero IAA destruction after 90 min). In contrast, the mixing resulted in no appreciable reduction in guaiacol oxidase activity (*A* 540 nm after 15 min = 0.395).

Combination treatment of peroxidase: H_2O_2 *followed by gel filtration*

A relatively rapid method of unmasking IAA-oxidase activity in crude *Vigna* extracts was developed using a combination of H_2O_2 and gel filtration treatment. The extracts were incubated with 5 mM H_2O_2 for 30 min at room temperature, then rapidly filtered through Sephadex PD-10 columns. The results of such treatment are illustrated in Fig. 1.

Filtration alone removed some of the Prs (presumably the low MW fraction), however, a lag lasting from one to several hr was still observed. No lag was observed in filtrates which had been treated with H_2O_2 first. The advantage of the filtration is that H_2O_2, low MW Prs and their oxidation products (quinones) are removed by the gel, and thus do not interfere with the IAA-oxidase assay. The treatment does cause some loss of guaiacol peroxidase activity.

The idea that the IAA-oxidase activity may be masked is consistent with the findings of Hoyle [14] who also suggested that high levels of inhibitor may move with the enzyme during purification.

Auxin protectors have been found in all plants examined thus far. Of interest to the peroxidase data are the 'A' class protectors (Pr-A) first identified in the Japanese morning glory [18]. These are extremely high MW substances which are partly proteinaceous [20]. In general, these substances tend to be associated with high MW proteinaceous preparations and could easily contaminate 'purified' fractions. In *Vigna* the Pr-A migrates more slowly through the dextran gel than the peroxidase, implying that it is a smaller molecule than the enzyme. In other plant species studied thus far, the reverse is true.

Auxin protectors appear to be derivatives, complexes or polymers of *o*-dihydroxyphenylpropanoids [21–23]. Their antioxidant properties vital to their ability to protect substrate from peroxidase-catalysed oxidation, are rapidly destroyed by H_2O_2 [24–26].

Protectors can also reduce partially oxidized substrate, as they do in the peroxidase-catalysed oxidation of glutathione [27]. This combination of effects may lead to significant changes in the kinetics of any assay involving freshly isolated plant peroxidases, thereby causing further confusion in determining the actual quantities of enzyme present in tissue. To complicate the matter still further, the oxidation products of the antioxidant *o*-phenols may be *o*-quinones—themselves powerful oxidants.

The data presented in this paper throw into question earlier reports which conclude that peroxidases and IAA oxidases are different enzymes, or represent different isozymes. That does not mean that we exclude the possibility that such isozymes exist. However, it seems more likely that the ability to oxidize IAA is one of the many capabilities intrinsic to peroxidase enzyme molecules.

EXPERIMENTAL

Plants. Cow peas, *Vigna sinensis* L., seedlings were grown in a greenhouse, or on a well-lit (daylight) laboratory window sill. The seedlings used were *ca* 14 days old and *ca* 10 cm tall.

Enzyme preparation. The plants were harvested intact and placed in a freezer for 17 hr at −14°. The roots were then excised and discarded. Shoots were ground up in a cold pestle and mortar using *ca* 50 g of tissue; the resulting slurry was filtered through cheesecloth, yielding *ca* 15 ml of liquid. The filtrate was then centrifuged for 10 min at 1300 *g*. The supernatant (3 ml) was immediately injected onto a chromatography column (length 40 cm, dia 2.6 cm) containing Octyl-Sepharose CL-4B at below 9°. The sample was eluted with 50 mM Pi buffer (pH 6.1) at a constant flow rate of 1 ml/min (2 ml fractions). The eluate was monitored at 280 nm.

The peroxidase assay mixture consisted of 30 mM Pi buffer pH 6.1, 0.1 mM DCP, 1 mM H_2O_2, 1 mM guaiacol. The enzyme sample was added to a final concn of 10%. After incubation at 30° for 15 min in a shaker H_2O bath the mixtures were read at 540 nm against a blank containing the mixture minus the enzyme prepn.

The IAA-oxidase assay mixture consisted of 30 mM Pi buffer (pH 6.1), 0.1 mM DCP, 0.1 mM Mn, 0.1 mM IAA. The enzyme sample was added to a final concn of 10%. The mixture was shaken at 30°. Aliquots (0.3 ml) were withdrawn at various time intervals, mixed with Salkowski reagent and read after 15 min in the colorimeter at 540 nm.

Ageing of enzyme. Enzyme fractions stored in the freezer at

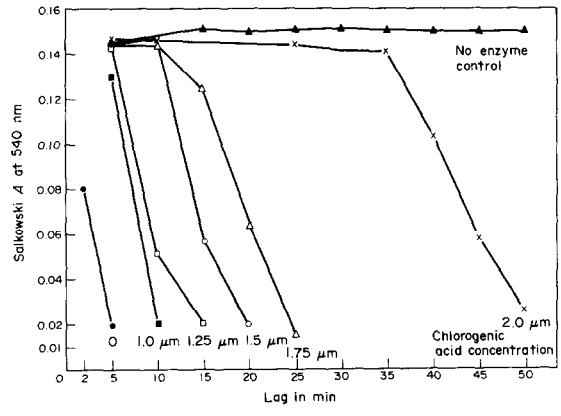

Fig. 2. Peroxidase-catalysed IAA oxidation as a function of Pr concentration.

– 14° were incubated for up to 46 hr in a mixture of 30 mM Pi buffer (pH 6.1), with and without peroxidase co-factors: 0.1 mM DCP and 0.1 mM Mn; then sample was added to a final concn of one fifteenth of the total vol. Following this incubation period, 2 ml samples were assayed for IAA-oxidase activity as above. IAA-oxidase activity was monitored for up to 20 hr.

Assay for Prs. The assay mixture consisted of 25 mM Pi buffer (pH 6.1), 0.1 mM DCP, 0.1 mM Mn, 0.1 mM IAA, 0.2, μg/ml horseradish peroxidase. The sample was added to a final concn of 10%. The mixture was shaken at 30°, 0.3 ml aliquots were withdrawn at intervals, mixed with Salkowski reagent and read after 15 min at 540 nm. The length of lag in the oxidation of IAA was taken as an indication of the amount of Prs present [28].

A more detailed quantitative relationship between the amount of Prs and lag time is provided in Fig. 2 showing the effect on lag time of increasing concn of chlorogenic acid, a presumed Pr of sunflowers [21].

Eliminating Prs from enzyme preparations. Crude *Vigna* extracts (2.5 ml) were incubated with 5 mM H_2O_2 for 30 min. At the end of this period they were rapidly filtered through Sephadex G25 PD10 columns. The extract was eluted with 50 mM Pi buffer (pH 6.1) and the eluate collected manually in 1 ml fractions. The bulk of the enzyme activity appeared in the 3rd fraction after the void vol.

REFERENCES

1. Saunders, B. C., Holmes-Stedle, A. G. and Stark, B. P. (1964) *Peroxidase: The Properties and Uses of a Versatile Enzyme and of Some Related Catalysts.* Butterworths, London.
2. Galston, A. W., Bonner, J. and Baker, R. S. (1953) *Arch. Biochem. Biophys.* **42**, 456.
3. Ray, P. M. (1958) *Annu. Rev. Plant Physiol.* **9**, 81.
4. Shannon, L. M. (1968) *Annu. Rev. Plant Physiol.* **19**, 187.
5. van der Mast, C. A. (1969) *Acta Bot. Neerl.* **18**, 620.
6. Sequeira, L. and Mineo, L. (1966) *Plant Physiol.* **41**, 1200.
7. Stafford, H. A. and Bravinder-Bree, S. (1972) *Plant Physiol.* **49**, 950.
8. Raa, J. (1971) *Physiol. Plant.* **24**, 498.
9. Raa, J. (1971) *Physiol. Plant.* **25**, 130.
10. Raa, J. (1973) *Physiol. Plant.* **28**, 132.
11. Stegel, B. Z. and Galston, A. W. (1967) *Science* **157**, 1557.
12. Srivastava, O. P. and van Huystee, R. B. (1977) *Phytochemistry* **16**, 1527.
13. Bastin, M. (1964) *Bull. Soc. R. Sci. Liège* **11**, 678.
14. Hoyle, M. C. (1972) *Plant Physiol.* **50**, 15.
15. Shinshi, H. and Noguchi, M. (1975a) *Phytochemistry* **14**, 1255.
16. Macnicol, P. K. (1973) *Phytochemistry* **12**, 1273.
17. Shinshi, H. and Noguchi, M. (1975b) *Phytochemistry* **14**, 2141.
18. Yoneda, Y. and Stonier, T. (1967) *Plant. Physiol.* **42**, 1017.
19. Endo, T. (1968) *Plant Cell Physiol.* **9**, 333.
20. Novak, J. and Galston, A. W. (1971) *Plant Cell Physiol.* **12**, 931.
21. Stonier, T. (1976) *Coll. Abst. of the Paper-Demonstr. 9th Int. Conf. of Plant Growth Substances*, (Pilet, P. E., ed.) p. 376. Lausanne, Switzerland.
22. Stonier, T., Singer, R. W. and Yang, H. M. (1970) *Plant Physiol.* **46**, 454.
23. Griffin, B. and Stonier, T. (1975) *Physiol. Plant.* **33**, 157.
24. Stonier, T. (1972) *Colloq. Int. C.N.R.S. No. 193—Les Cultures de Tissus de Plantes* (Hirth, M. L. and Morel, G., eds.) p. 423.
25. Stonier, T., Yoneda, Y. and Rodriguez-Tormes, F. (1968) *Plant Physiol.* **43**, 1141.
26. Stonier, T., Hudek, J., Vande-Stowe, R. and Yang, H. M. (1970) *Physiol. Plant.* **23**, 775.
27. Stonier, T. and Yang, H. M. (1973) *Plant Physiol.* **51**, 391.
28. Stonier, T. and Yoneda, Y. (1967) *Ann. N.Y. Acad. Sci.* **144**, 129.