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Studies on auxin protector substances, IAA-oxidase and peroxidase in cotyledons of *Pharbitis nil*

Josef Novák and Arthur W. Galston

School of Forestry and Department of Biology, Yale University,
New Haven, Connecticut 06520, U. S. A.

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The nature of macromolecular "auxin protector substances" causing lag periods rather than inhibition in the rate of IAA oxidation was reinvestigated. Three different peaks were separated by Sephadex gel filtration; each was then examined by means of enzymatic (IAA oxidase, peroxidase) and electrophoretic techniques and correlated with the activities of both enzymes and with zymogram patters. The auxin protector activity of the high molecular weight fractions increased after high temperature treatment. On the basis of experiments involving dialysis and chromatography before and after heating, auxin protectors appear to be complexes of macromolecules with small molecules.

The mechanisms through which indoleacetic acid (IAA) level is regulated have remained obscure although some attention has been paid to enzymes of synthesis (*1*), conjugation (*2*, *3*) and destruction (*4*). One new contribution to an understanding of the control of destruction rates has been the discovery of so-called macromolecular "auxin protectors" by Yoneda and Stonier (*5*) and Stonier and Yoneda (*6*). The sparing action of these substances on auxin destruction is based on the induction of a lag period rather than on an alteration of the rate of IAA oxidation. The changes in level of auxin protectors during development and the pattern of their distribution in various plant parts (*7*) makes reasonable the suggestion that IAA-induced processes may be controlled indirectly by the level of these substances. Many low molecular weight substances are considered to function in a similar way; these include various phenolic compounds (*4*, *8–10*), flavonoids (*11*), manganese (*12*, *13*) and plant acids working through inhibition of catalase (*14*). Hillman and Galston (*13*) have shown that the control of the rate of IAA oxidation by Mn^{++} and phenols depends on the molar ratio of these compounds; similarly Stonier, Rodriguez-Tormes and Yoneda (*15*) suggested that manganese promotes IAA oxidation through inactivation of the macromolecular "auxin protectors". Our experiments lead us to the belief that the auxin protector systems of *Pharbitis nil* are actually low-molecular weight effective compounds bound to inactive macromolecules.

Materials and methods

Seeds of *Pharbitis nil* Choisy (hereafter designed as wild type—WT) and *P. nil* var. Murasaki (M) were obtained through a generous gift of Dr. W. D. Mitchell

of the Du Pont Experimental Station, Wilmington, Del. and were used throughout this study. To achieve maximum germination, the seeds were treated for 5–90 min (depending on their water content) with concentrated sulphuric acid, kept under 30°C running water over night, and germinated on wet sand in Petri dishes for 24 hr in darkness. Finally they were planted in small pots filled with vermiculite and cultivated under continuous light at 23°C. The commercially obtained plant nutrient "Hyponex" further enriched with chelated iron was used. Cotyledons of different ages were excised and used immediately for extraction of protectors and enzymes.

1. *Extraction of auxin protectors.*

The methods of Stonier *et al.* (*15*, *16*) were used. One g fresh weight of tissue was ground per ml cold 20 mM phosphate buffer, pH 6.1 in a chilled mortar with sand. The homogenate was squeezed through 2 layers of cheese cloth and centrifuged at $6000 \times g$ for 15 min in a refrigerated centrifuge. The supernatant fluid was either further processed or immediately applied to the chromatographic column. All procedures were performed in a cold room at 2–4°C.

2. *Fractionation.*

Gel filtration was performed by means of a Sephadex G-50 column (14×300 mm). The flow rate (20 mM phosphate buffer, pH 6.1) was 0.37 ml/min, and approximately 4.0 ml fractions were collected. The fractions were collected in the cold room, frozen as soon as possible, and kept in a deep-freeze until use, but no longer than several days.

3. *Reaction mixture.*

The reaction mixture contained 0.2–1 ml of a particular extracted fraction, 0.1 mM 2,4-dichlorophenol, 0.1 mM manganese chloride, 0.1 mM of IAA and horseradish peroxidase (HRP; Nutritional Biochemicals Co.) in a final concentration of 0.2 μg/ml. Substances were added to the 0.02 M potassium phosphate buffer, pH 6.1 in the order stated. The final volume was 10 ml, and the reaction permitted to proceed in 25 ml Erlenmeyer flasks placed in Dubnoff metabolic shaking incubators at 32°C. Where endogenous IAA-oxidase determinations were to be made, the HRP was omitted.

4. *Colorimetric assay of IAA oxidation.*

At given intervals after the start of the reaction 1 ml aliquots were removed and mixed with 2 ml of Salkowski reagent (*17*). The color developed was measured after at least 20 min in a Bausch and Lomb Spectronic-20 colorimeter; a mixture of 1 ml of distilled water with 2 ml of Salkowski reagent was used as a blank.

5. *Peroxidase assay.*

The reaction mixture contained 0.1 M phosphate buffer, pH 5.8, guaiacol 15 mM and hydrogen peroxide 15 mM. After addition of an aliquot of a particular fraction, the reaction was followed by recording the optical density at 470 nm every 15 sec for 2 min in a Beckman-Gilford spectrophotometer. The average

difference between readings in the linear phase of reaction was used for determination of peroxidative activity of fractions.

6. *Dialysis and heating treatments.*

Dialysis of the supernatant obtained after the centrifugation of extract was against 1 liter of 20 mM phosphate buffer for 24 hr at 5°C. Particular fractions obtained from gel filtration were dialyzed for shorter times as stated below. Heating was performed in water bath at 95–98°C for 5 min. Tubes with 1 ml or less of the particular fraction were immediately cooled and used for assay.

7. *Electrophoresis and gel scanning.*

Starch gel electrophoresis of peroxidases was performed as already described (*18*). The zymograms were photographed with Polaroid instant film; the gels were also quantitatively scanned in the spectrophotometer equipped with a linear transport assembly, and the results automatically recorded. The activity of particular isoperoxidases could be determined by calculation of the area under the curves.

Results

1. *Distribution of auxin protector substances, peroxidase and IAA oxidase in fractions after gel filtration.*

Usually 3 peaks of auxin protector activity were obatined after gel filtration of extracts of 10-day old WT cotyledons, confirming Yoneda and Stonier (*7*) (see black bars in Fig. 4).

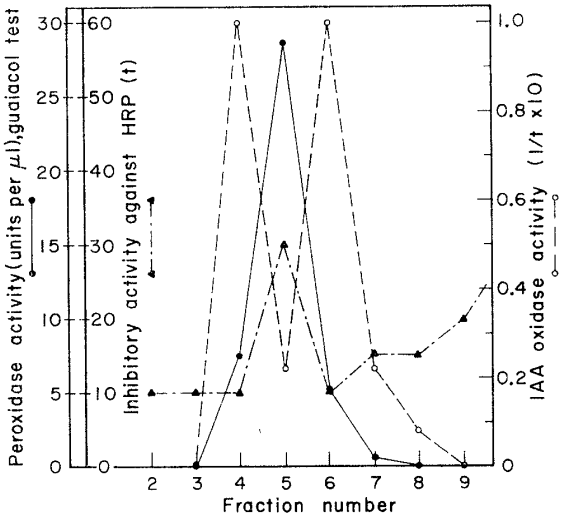

Fig. 1. *Distribution of peroxidase, IAA oxidase and auxin protector activity in extract of 10-day old WT cotyledons after gel filtration.* 3 g of cotyledons were ground in 3 ml of phosphate buffer, pH 6.1. Fractionation performed at cold temperature. 4 ml fractions were immediately frozen and assayed within 1 week. 1 ml of particular fraction assayed for IAA oxidase. 0.2 ml for inhibitory activity (against HRP mediated IAA oxidation). T=time required to reach OD=0.1 in min.

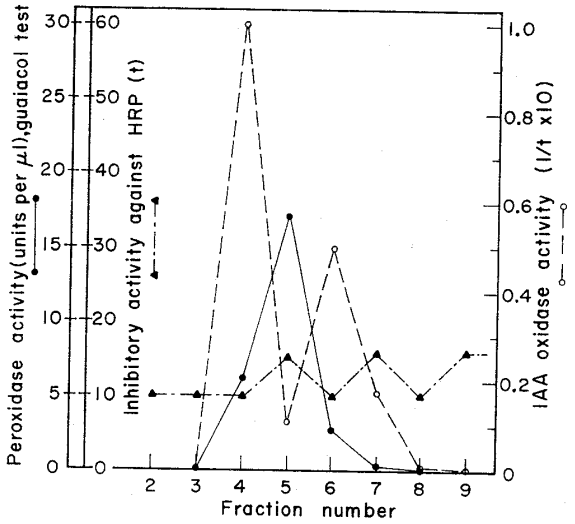

Fig. 2. *Distribution of peroxidase, IAA oxidase and auxin protector activity in some of the extracts of 10-day old M cotyledons after gel filtration.* Extraction and assay procedure same as already described under Fig. 1.

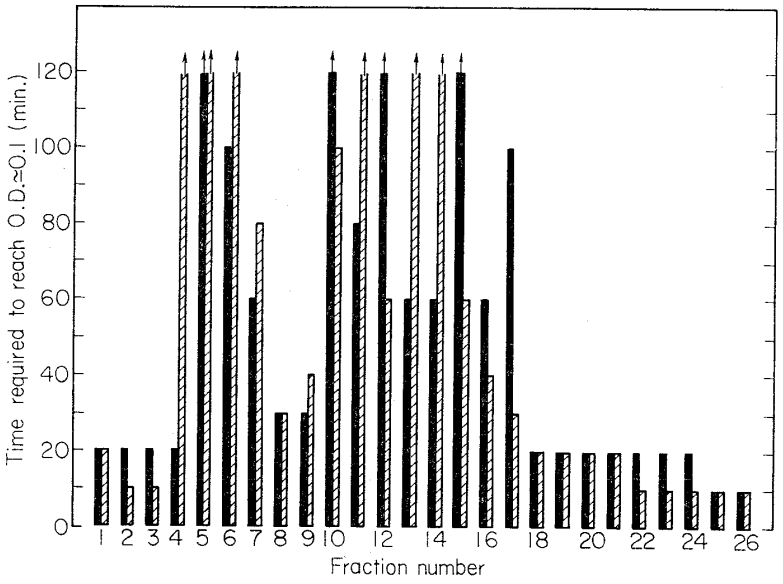

Fig. 3. *Effect of dialysis on the distribution of IAA protector substances in extract of WT variety.* (■) control (not-heated), (▨) heated. Dialysis was performed as described in **Methods** for 24 hr. Contents of dialysis bag were then immediately applied to the Sephadex column and only 3 ml fractions collected. All procedures were carried out below 5°C and fractionation itself under 1–2°C. Heating was done after gel filtration and was performed with every fraction as described in **Methods**. 0.5 ml aliquots used for assay. Dialysate (1 liter) was concentrated under vacuum at 30°C to a volume of 17.4 ml; however, 0.5 ml and 1 ml portions of it did not cause any delay of IAA destruction. In several cases the conditions were such that even after 120 min of reaction the IAA was not destroyed by HRP or endogenous IAA oxidase. Such cases are indicated by arrows over some bars, since the reaction was not measured beyond 120 min.

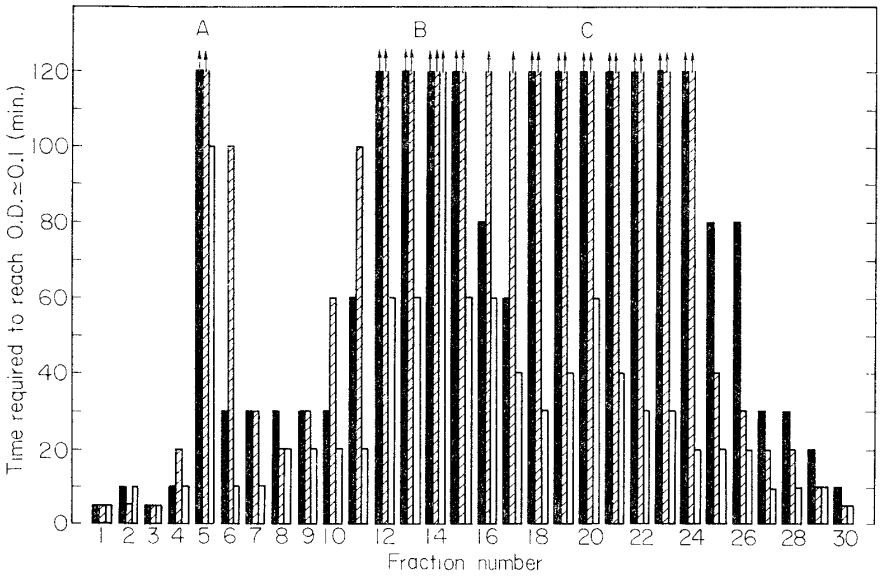

Fig. 4. *Effect of heating on the auxin protector activity of fractions of crude homogenate taken off the Sephadex column.* 4 ml fractions were collected, ■ and □: 0.5 and 0.3 ml aliquots of non-treated fractions assayed (control), ▨: 0.5 ml, heated. (Variety WT.)

Peroxidase, IAA oxidase and IAA-protector substance activities were determined in the highest molecular weight active fractions (Protector A) taken from the column; results are presented in Fig. 1 and 2 for the WT and M varieties, respectively. It is clear that the IAA oxidase activity in both cases does not follow the peroxidase activity; actually the peak of peroxidase activity occurs in fractions (No. 5) in which the IAA-oxidase activity is very low. It might be suggested from Fig. 1 that the inhibitory substances (in this case Protector A) must be present in high concentrations to inhibit IAA oxidase and that this inhibition is specific in comparison with the peroxidase activity. Nevertheless in some of the experiments using 10 day-old cotyledons of M variety the pattern shown in Fig. 2 has been obtained, revealing an apparent lack of the auxin protector A activity in the critical No. 5 fraction. We believe that the inhibitory substances are not always detectable under conditions of assay involving inhibition of IAA oxidation by HRP. This hypothesis will be examined later, when the heating experiments are discussed.

2. *The effect of dialysis and high temperature treatments on the properties of inhibitory substances.*

When crude extracts from 10 day-old WT cotyledons are dialyzed for 24 hr in the cold room prior to separation on the Sephadex column, all auxin protector substances beyond fraction 17 are lost (Fig. 3, compare black bars in Fig. 4). Furthermore changes in protector activity occurs as a result of heating the residues of dialysis; some fractions decrease and others increase in activity. The increase of inhibitory activity after heat treatment was confirmed in an experiment in which nondialyzed crude extract was used for fractionation (Fig. 4). Except for the

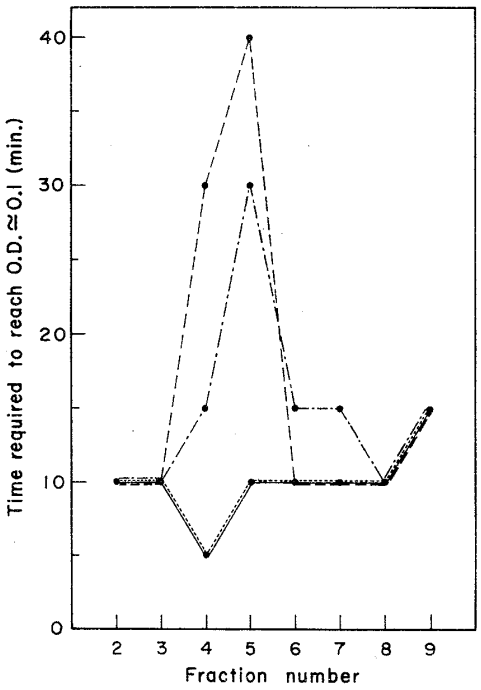

Fig. 5. *Effect of similar treatments as described in Fig. 3 and 4 on the inhibitory substances of high molecular fractions extracted from variety M.* Extraction and assays same as in Fig. 1. Then 0.3 ml was used for regular inhibitor assay (——), 0.5 ml heated (see **Methods**) and 0.3 ml aliquots then used (– – –), 0.3 ml dialyzed for 3 hr against 0.02 M phosphate buffer (at 1°C) with several changes (•••); 0.5 ml heated, then dialyzed as above and 0.3 ml aliquot used (–•–•–).

smallest molecular weight fractions (high numbers), heating increased the inhibitory activity.

As was already pointed out in some experiments, the highest molecular weight protector fraction seemed to be absent from 10 day-old cotyledons of M variety, although it was always present in WT (see Fig. 1 and 2). The heat technique was employed to see whether any latent protector activity resided in appropriate eluates from M extracts. Fig. 5 shows that there is initially very low protector activity, which is unaffected by dialysis. After heating, such activity appears, and is not completely dialyzable.

3. *Isoenzymic pattern of peroxidases in peroxidase and IAA-oxidase rich fractions.*

Electrophoretic studies were conducted to determine the relationship among peroxidase isoenzymes, IAA oxidase and inhibitory compounds. Three conclusions could be drawn from these experiments: 1. In the different fractions obtained by means of gel filtration different isoenzymes of peroxidase appear, as revealed by starch gel electrophoresis, 2. Total peroxidase activity of the homogenate is always lower than the total activity of peroxidase isoenzyme measured from electropherograms, 3. The activity of IAA oxidase does not follow any of peroxidase isoenzymes. Fig. 6 depicts an electropherogram of the peroxidase

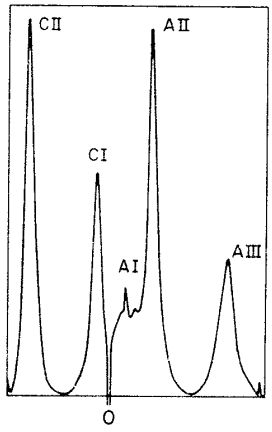

Fig. 6. *Peroxidase isoenzymes obtained after electrophoresis of the crude extract from 10-day old cotyledons of M variety.*

isoenzymes extracted from the 10 day-old cotyledons of M variety. Basically two cationic (CI and CII) and three anionic (AI, AII and AIII) isoperoxidases were present in this stage of development. WT gave similar results. If the entire supernatant was first chromatographed on a Sephadex G-50 column and each fraction then separated by electrophoresis the results in Table 1 are obtained. It is clear that the earliest (largest mol. weight) fractions are high in anionic isozymes and that the ratio of cationic : anionic isoperoxidase activity increases progressively with decreasing size of the fraction. Isoperoxidases AII and AIII

Table 1

Protector activity, peroxidase activity and peroxidase isoenzyme pattern of fractions obtained by means of Sephadex G-50 chromatography

Fraction	CI	CII	ΣC	AI	AII	AIII	ΣA	$\frac{\Sigma C+}{\Sigma A}$	$\frac{\Sigma C}{\Sigma A}$	Peroxidase (guaiacol)	Protector activity (min)	Heated protector (min)
	(in arbitrary units per μl of the original fraction)											
4	10	—	10	2	12	7	21	31	0.47	0.99	15	15
5	230	340	570	430	2120	480	3030	3600	0.18	13.29	>120	>120
6	200	490	690	280	920	470	1670	2360	0.41	8.58	40	90
7	46	145	191	19	39	22	80	271	2.38	0.54	20	40
8	37	77	114	15	10	—	25	139	4.56	0.11	20	90
9	27	55	82	12	—	—	12	94	6.33	0.00	15	20
10	27	55	82	5	—	—	5	87	16.40	0.30	30	90
11	17	60	77	5	—	—	5	82	15.40	0.69	40	90
12	17	50	67	2	—	—	2	69	33.33	0.30	90	90
13	7	17	24	—	—	—	—	24	24.00	0.24	120	120
14	7	—	—	—	—	—	—	7	7.00	0.00	40	90
WS	1110	1900	3010	550	2220	1030	3800	6810	0.79	21.84	>120	>120

3 g of 10-day old cotyledons of M variety were ground in buffer in the cold. Supernatant fraction from the centrifugation were immediately applied to the column. Portions of the crude extract (WS) and of each fraction were used for enzymatic and electrophoretic assays, described in methods. The last two columns indicate the time required for HRP to destroy enough IAA to bring the OD of the Salkowski reaction color down to 0.1. Endogenous IAA-oxidase activity was observed only in fractions 4 through 8 with peaks in fractions 4 and 6 (similarly to Fig. 1 and 2).

are present only in higher molecular weight fractions. The ratio of CI/CII is relatively constant in all fractions (about 0.39). In these preliminary experiments no further attempts have been made to correlate the content of auxin protector substances with the quality and quantity of bands on electropherograms.

Discussion

Recently, several reports dealing with high molecular weight enzyme inhibitor have appeared in addition to those of Yoneda and Stonier. Bradshaw, Chapman and Edelman (*19*) found such inhibitors for invertase, Ridge and Osborne (*20*) confirmed the existence of high molecular weight inhibitor of peroxidase, Lane and King (*14*) established a model for a catalase-induced lag period in IAA destruction by IAA-oxidase while Phipps (*21*) had already described a proteinaceous inhibitor of the same enzyme in 1965. In most of these cases, the high molecular weight compounds were heat-labile. Our own results suggest that while some inhibitory materials may be heat labile and dialyzable other dialyzed high molecular weight fractions show an increased partially dialyzable inhibitory activity after heating. This suggests that protector substances are in fact small molecules bound to the larger, presumably proteinaceous heat labile carriers.

From Table 1 and Fig. 1 and 2 it appears that IAA oxidase possesses a broader molecular weight distribution than other peroxidase isoenzymes. This might be partially due to the presence of auxin protector substances in the zymograms, as suggested by Yoneda and Endo (*22*). We suspect that similar interference exists in our electrophoretic experiments. The almost complete absence of separable bands in fraction No. 4 (see Table 1) and the high efficiency of the endogenous IAA oxidase of the same fraction in the reaction mixture could be explained by the coincidence of auxin protectors and isoperoxidases in gels.

The present view that IAA oxidase is one of the peroxidase isoenzymes has received much recent attention. Stutz (*23*), and Galston, Lavee and Siegel (*18*), proposed a model according to which the enzyme contains two active sites, one for IAA oxidation, the other for peroxidase activity. There is evidence both for (*24*) and against (*25*) the view that the protein of peroxidase has IAA oxidative activity but no peroxidative activity. Janssen (*26*, *27*) suggested that not only peroxidase and IAA oxidase but also polyphenoloxidase activities from pea roots are due to one enzyme. Some authors, however, have established that some preparations of enzyme have only IAA oxidase (*28*, *29*) and no peroxidase activity. Our results (Table 1, Fig. 1 and 2) incline to a similar conclusion.

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