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SYNTHESIS OF ISOPEROXIDASES IN LENTIL EMBRYONIC AXIS

A. A. KHAN, TH. GASPAR, C. H. ROE, M. BOUCHET and M. DUBUCQ

New York State Agricultural Experiment Station, Cornell University Geneva, New York, U.S.A. and Laboratoire de Biologie végétale, Institut Van Beneden, Université de Liège, Liège, Belgique

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Abstract—Isoperoxidases were synthesized *de novo* in the embryonic axis of lentil. Nearly 50% decay of *m*RNA for peroxidase occurred in 3 hr. Detachment of the embryonic axis from the cotyledons caused a 2–3-fold increase in the production of peroxidase and a decrease in the lag period for enzyme synthesis. This increase in enzyme synthesis appeared to be due largely to a sharp rise in the intensity of 5–7 cathodic isoperoxidases as revealed by starch gel electrophoresis. Two anodic isoperoxidases appeared with greater intensity in the attached axis.

INTRODUCTION

PROFOUND changes in metabolic activities in plant tissues occur when they are subjected to wounding,[1–7] infection by pathogens,[4,8–10] aging[7,11–19] or other conditions of stress. Under these conditions induction of several enzymes has been reported. Among the enzymes most studied are peroxidase,[7,10,12,20] invertase[3,13–19,21] and phenylalanine

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ammonia-lyase.[22–29] In some instances changes in enzyme (or protein) activity have been shown to be a result of *de novo* synthesis.[10,15,20,30] In most cases tissue slices from tubers, stems and roots have been used.

We report here the synthesis of isoperoxidases (E.C. 1.11.1.7) in the lentil embryonic axis grown attached (*in situ*) and detached from the cotyledons. It will be shown that detachment of the axis from the cotyledons induces an increase in the synthesis of peroxidase. This increase appears to be due to changes unrelated to those required for the synthesis of the enzyme in the axis during germination *in situ*. It will be shown that peroxidases are synthesized *de novo*.

RESULTS

The time course of synthesis of total peroxidase in lentil embryonic axis grown attached (*in situ*) and detached is shown in Fig. 1. It may be noted that beginning with a lag phase there is a sharp increase in the production of the enzyme with time up to about 48 hr of growth (Fig. 1). Thereafter the enzyme synthesis levels off. However, the detachment of the

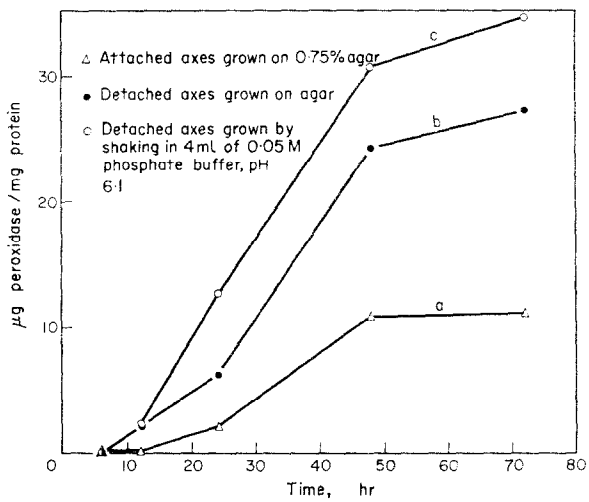

FIG. 1. PEROXIDASE SYNTHESIS IN EMBRYONIC AXES GROWN ATTACHED (*in situ*) AND DETACHED FROM COTYLEDONS.

axis shortens this lag period for enzyme synthesis from *ca.* 10–12 hr to *ca.* 6–8 hr and causes an increase in enzyme production (Fig. 1b). This increase is *ca.* 2–3-fold after 24 and 48 hr of growth. A further increase in peroxidase synthesis, although not necessarily a decrease in the lag period, is observed when excised axes are grown in a shake culture (Fig. 1c). An

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increase in enzyme activity of up to 2-fold in 24 hr is found in axes grown by shaking, over those grown without shaking (Fig. 1b, c). There is a profound difference in the growing ability of the two axes. The detached axis grows very slowly (Fig. 2). An inverse relationship between growth potential and peroxidase activity has been emphasized by Galston and his coworkers.[12,31] There was no decrease in the level of enzyme activity once formed suggesting that the enzyme is stable and not subject to decay or rapid turn-over as has been observed for some other enzymes.[32–35]

In view of the pronounced difference in the enzyme synthesizing capacity of the attached and the detached embryonic axis, it was considered desirable to investigate the ontogeny of isoperoxidases in the two systems. Previous work[36] had shown that up to 15 isoperoxidases from 6-day-old lentil roots can be resolved by starch gel electrophoresis. In the present studies the time course of development of the multiple forms of peroxidases in attached and detached lentil axes is shown in Fig. 3. Starting from 1 or 2 faintly visible cathodic peroxidases after 1–2 hr of soaking, the number in detached axes increases to 8[6 cathodic(−) and 2 anodic(+)] after 12 hr, 14 (8−, 6+) after 24 hr and 15 (8−, 7+) after 48 hr. The resolution of cathodic peroxidases varied from 6–8. In attached axes the numbers were 6 (4−, 2+) after 12 hr, 13 (7−, 6+) after 24 hr and 14 (7−, 7+) after 48 hr.

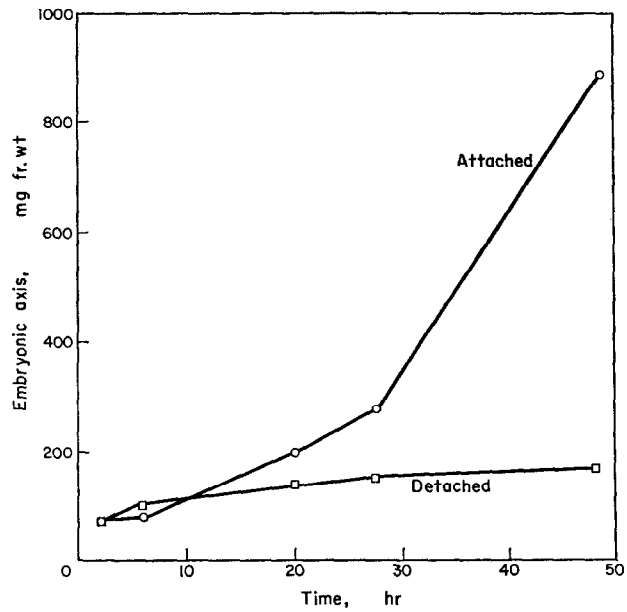

FIG. 2. THE INCREASE IN fr. wt OF THE LENTIL EMBRYONIC AXES (AV. 20) GROWN ATTACHED AND DETACHED FROM THE COTYLEDONS ON 0·75% AGAR IN THE DARK AT 26°. AXES GROWN *in situ* (ATTACHED) WERE DETACHED JUST PRIOR TO WEIGHING.

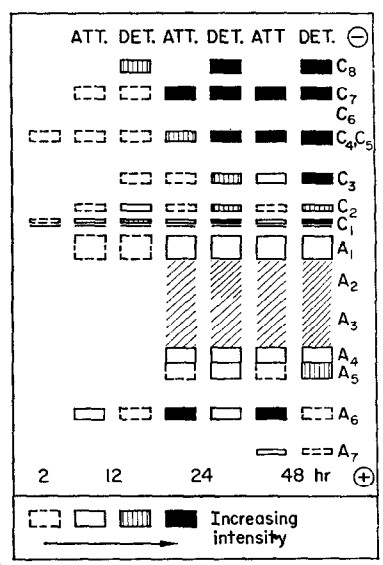

FIG. 3. ISOENZYMES OF PEROXIDASE FROM ATTACHED AND DETACHED AXES RESOLVED BY STARCH GEL ELECTROPHORESIS. THE ISOENZYMES IN EACH CASE WERE FROM AN EXTRACT EQUIVALENT TO 58 mg OF EMBRYONIC AXES.

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All cathodic isoenzymes from detached axes, with the exception of one (C_7), appeared with greater intensity than their counterpart from the attached axes. The isoenzyme C_7 appeared with equal intensity in both cases. 2 anodic isoenzymes (A_6, A_7) showed up with greater intensity in attached axes while the reverse held true in the case of 1 anodic isoenzyme (A_5). Thus there are some qualitative differences in the pattern of isoperoxidase development in the two axes. However, the increased activity in total peroxidase due to detachment appears to be clearly due to an increase in 5–7 cathodic isoenzymes.

TABLE 1. EFFECT OF APPLICATION OF INHIBITORS OF RNA SYNTHESIS ON PEROXIDASE SYNTHESIS DURING DEVELOPMENT OF DETACHED EMBRYONIC AXIS. PERIOD OF TREATMENT WAS 44 hr

Hour after germination at which axis transferred from water to inhibitor	Peroxidase synthesis (μg/mg protein)	
	6-methylpurine	Actinomycin-D
0	0·74	5·70
6	1·15	4·52
18	22·8	35·4
24	25·3	46·0
44	—	43·7

We next determined the nature of the process leading to increased enzyme activity. Is the enzyme synthesized *de novo* or is it formed as a result of activation of pre-existing proteins? Actinomycin D, 6-methylpurine and cycloheximide, specific inhibitors of RNA and protein synthesis,[37–39] were employed. The effects of inhibitors of RNA synthesis applied at various times on the development of total peroxidase in detached axis are shown in Table 1. Actinomycin D given at 0 time or during the lag period was active in inhibiting enzyme synthesis by nearly 90–95%. However, it was essential to apply the inhibitor in the cold with shaking to obtain maximum inhibition of peroxidase synthesis (Table 2). 6-Methylpurine completely inhibited peroxidase synthesis. Unlike actinomycin D, this inhibitor appeared to suppress enzyme synthesis even when applied after 24 hr of growth. This would indicate that at this stage of growth 6-methylpurine reaches the site of action much more rapidly than actinomycin D. Actinomycin D given after the lag period (12 hr) inhibited peroxidase synthesis appreciably (see Fig. 5) but had little effect when given after 18 hr of growth (Table 1). These results would suggest that RNA synthesis is required for peroxidase formation and that RNA synthesis is completely limiting for peroxidase synthesis only in the initial stages of growth of the axis.

The effects of cycloheximide on the development of peroxidase in detached axes is shown in Fig. 4. It completely suppressed peroxidase synthesis when given at 2, 8 and 24 hr of growth. There was no sign of decay of this enzyme following suppression of enzyme activity. These results strongly suggest that the enzyme is synthesized *de novo* and is not formed as a result of activation of existing protein.

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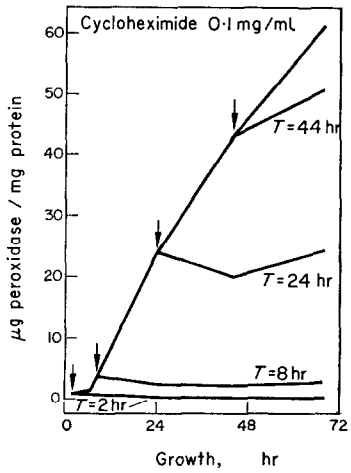

FIG. 4. THE EFFECTS OF CYCLOHEXIMIDE (60 μg/ml) APPLIED AT DIFFERENT TIMES ON THE SYNTHESIS OF PEROXIDASE BY DETACHED EMBRYONIC AXES. AXES WERE GROWN ON 0·75% AGAR IN THE DARK AT 26°.

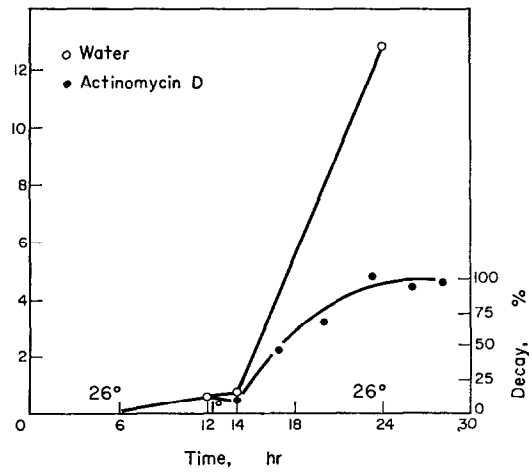

FIG. 5. THE RATE OF DECAY OF *m*RNA FOR PEROXIDASE IN DETACHED AXES. THE AXES (20/DETERMINATION) WERE GROWN BY SHAKING. ACTINOMYCIN D (60 μg/ml) WAS APPLIED FOR 2 hr (BETWEEN 12 AND 14 hr) AT 1° BY SHAKING ON AN ICE BATH.

The effects of inhibitors at 60 μg/ml on the development of isoenzymes in detached axis were also investigated. Cycloheximide given at the beginning or during the lag period completely suppressed the normal development of the isoenzymes. Actinomycin D and 6-methylpurine similarly given inhibited the development of 4–5 cathodic isoenzymes. 6-Methylpurine was more active than actinomycin D in suppressing the development of anodic isoenzymes. In this experiment actinomycin D was applied at 0° without shaking. This inhibitor is more effective when applied by shaking in the cold (Table 2). After 18 and 24 hr neither cycloheximide nor RNA synthesis inhibitors had any effect on the pattern of isoperoxidases. The embryonic axes grown *in situ* were similarly unable to synthesize various peroxidases, in the presence of the inhibitors. From these results it can be concluded that various peroxidases are synthesized *de novo*. It is not unlikely however that the embryonic axis contains some preformed messengers which code for some of the peroxidases. An interesting example of preformed-messengers in mature embryos is found in wheat and cotton embryos.[40,41] The contribution of these peroxidases to total activity of the enzyme however, appears negligible (Tables 1 and 2).

We followed the method of Gayler and Glasziou[20] in an attempt to determine the half-life of *m*RNA or *m*RNAs which code specifically for the peroxidases. Actinomycin D was applied at 12 hr following imbibition of the detached axes at which time the *m*RNA(s) for the peroxidases had been synthesized. Subsequently the rate of decay of these RNA(s) was followed at close intervals. The results are shown in Fig. 5. It may be seen that the initial capacity (100%) of the RNA to synthesize peroxidase is gradually lost. It takes about 3 hr to reduce by 50% the capacity of the RNA (s) to synthesize peroxidases. This time is slightly more than that for the peroxidase of sugar cane stem tissue which had a half-life of 1·5–2 hr.[20]

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TABLE 2. EFFECT OF MODE OF APPLICATION OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON PEROXIDASE SYNTHESIS BY DETACHED EMBRYONIC AXIS

Treatment	Peroxidase (μg/mg protein)		
	24 hr	48 hr	62 hr
Actinomycin D (agar)	3·3	10·9	—
Actinomycin D (shaking*	2·4	5·0	—
Actinomycin D(shaking)†	1·2	1·6	2·0
Cycloheximide (agar)	0·4	0·4	0·3
Cycloheximide(shaking)*	0·1	0·1	0·1
6-Methylpurine(shaking)†	—	—	0·1
Water	10·5	45·0	48·1

* Embryonic axes detached from embryos after 2 hr imbibition of seeds in water at 26°, axes transferred to inhibitor (60 μg/ml) for 2 hr at 1°, then returned to 26°.

† Axes detached from embryos after 4 hr at 1–2°, transferred to inhibitor (60 μg/ml) for 2 hr at 1°, then the contents returned to 26°.

DISCUSSION

Peroxidase and other enzymes have been shown to increase dramatically in plant tissues and organs under conditions which impose restriction on growth, notably injury and infection. In the present studies although peroxidase is synthesized in the embryonic axis of lentil *in situ*, the level of activity increases considerably as a result of detachment of the axis.

The synthesis and the rate of formation of an enzyme may be controlled at the level of transcription or translation. Although the data do not rule out some regulation at the level of protein synthesis, an obligatory requirement of RNA synthesis for enzyme formation is shown by the use of specific inhibitors of RNA synthesis, namely actinomycin D and 6-methylpurine. Half-life of *m*RNA(s) for peroxidase is about 3 hr. These results suggest that peroxidases are synthesized *de novo* on unstable *m*RNAs.

There were profound differences in the rate of formation of total peroxidase as well as in the rate of development of individual isoenzymes in the attached and detached axes. However, there was little difference in the total number of isoenzymes during 24–48 hr of development of the two axes. How can these differences be explained if we invoke transcriptional control? It is now believed that genomic transcription can come about in one of several ways in eukaryotes.[34] The control could be a negative one or a positive one. It is possible that a number of isoenzymes are preferentially synthesized in detached axes as a result of selective transcription of one or more portions of the genome. Some factor or factors present in the cotyledon partially represses the synthesis of these isoenzymes in attached axes; absence of cotyledons causes derepression. This explanation is supported by the observation that in attached axes most of the isoenzymes, notably cathodic ones, develop with extremely low intensity. An equally plausible explanation for the differential rate of peroxidase synthesis would be that some factor is released in the axis as a result of its detachment from the cotyledons and this causes derepression of the repressed sites on the genome. This explanation is supported by the fact that several hormones such as indoleacetic

acid,[12,44] kinetin,[12] abscisic acid (unpublished data), gibberellin[45] and ethylene[42,43] induce specific changes in isoenzyme patterns of peroxidase. The repression hypothesis of *de novo* peroxidase formation is supported by a recent observation of a macromolecular repressor in tobacco pith tissue which is unable to form new peroxidases.[46]

A plant embryo or an embryonic axis can be regarded as a miniature plant. They are excellent for system analysis. Seeds such as lentils where cotyledons and embryonic axis make up almost the entire seed, an understanding of their relative role in growth and metabolism would be invaluable. However, detaching cotyledons from the axis causes injury in the axis at two points where cotyledons join the axis (cotyledonary node). As a consequence considerably higher level of peroxidases are synthesized in detached axes compared to the one grown *in situ*. This increased activity is rather specific and probably unrelated to the process of germination (Figs. 1 and 3). We believe that these studies may provide a framework for studying other enzymes and other biochemical events governing two separate plant processes—wounding and germination.

EXPERIMENTAL

Lentil (*Vicia lens*) seeds were soaked for 5 min in 1% NaClO, washed and then stirred for 1–2 hr in distilled water. Embryos were gently squeezed out of the seed coats. Embryonic axes from these embryos were obtained by detaching the two cotyledons. In some experiments with actinomycin D, the embryos and the embryonic axes were obtained from seeds which were stirred for 3–4 hr in the cold (1–2°).

The embryos or the axes were grown on 0·75% bacto-agar or by shaking in 4 ml of 0·05 M phosphate buffer, pH 6·1, with or without appropriate inhibitors, at 26° in the dark. Parallel determinations of total peroxidase and its isoenzymes were made at different periods of growth in axes grown attached or detached from the cotyledons. To determine the level of peroxidase in the axes, *in situ*, the axes were deached from the cotyledons just prior to their processing for the enzyme. 20 axes were homogenized in 0·5–2·0 ml of 0·05 M phosphate buffer, pH 7·8 and the homogenate spun at 25 000 g. In some preparations requiring separation of isoperoxidases, 140 mg of axes of various ages were homogenized in 0·5 ml of the buffer. The supernatant was used for the assay of peroxidase,[47] isoperoxidases and proteins.[48] Isoperoxidases were separated by starch gel electrophoresis performed by the method of Scopes.[49] The isoenzymes were revealed using *o*-dianisidine as a substrate.[50] Total peroxidase was quantitated using horseradish peroxidase (Worthington).

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