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HORMONAL CONTROL OF PEROXIDASE ACTIVITY IN GERMINATING MUNG BEAN COTYLEDONS

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Key Word Index—*Phaseolus aureus*; Leguminosae; mung bean cotyledons; regulation of peroxidase activity; auxin, ethrel and abscisic acid.

Abstract—A rise in peroxidase activity (22–35 fold) was observed in excised mung bean cotyledons 72 hr after germination. The enhanced enzyme activity was associated with the appearance of six new multiple forms of peroxidase. Supraoptimal concentration of auxin (IAA, 10^{-4} M, 10^{-3} M) brought about additional promotion (2–4 fold) of peroxidase activity over the water controls. Substitution of auxin by ethrel failed to enhance peroxidase activity, suggesting that the auxin response is not an ethylene mediated phenomenon. Gibberellic acid and kinetin also failed to alter the peroxidase activity and its multiple forms in excised cotyledons. However, cotyledons treated with abscisic acid (ABA, 100 μg/ml) showed a strong inhibition (87%) of peroxidase activity and this response was substantially counteracted by auxin. Actinomycin D and cycloheximide severely reduced peroxidase activity both in controls and auxin-treated cotyledons. This indicated the requirement of transcription and translation for the enhancement of peroxidase activity in germinating cotyledons.

INTRODUCTION

In several plant tissues, ethylene has been implicated in the regulation of peroxidase activity. Ethylene enhanced peroxidase activity in cotton leaf [1], mango slices [2], sweet potato root discs [3], potato stolon tips [4], pea epicotyl segments [5] and intact tobacco plants [6]. However, in detached leaves of tobacco [7], ethylene was responsible for the inhibition of peroxidase activity and in carrot tissue, the injury-induced peroxidase activity was not affected by ethylene [8].

Auxin is also reported to enhance peroxidase activity. Since the high concentration of auxin promoted the release of ethylene, it was suggested that auxin-stimulated peroxidase activity is an ethylene-mediated phenomenon [9, 10]. Auxin treatment of excised segments of mung bean seedlings [11] and pea shoots [12] resulted in the stimulation of ethylene production. Sakai and Imaseki [13] reported enhanced incorporation of ^{14}C-methionine into ethylene in IAA-treated mung bean seedlings. In cotton plants, the early appearance of increased ethylene in response to auxin was responsible for the stimulation of peroxidase and IAA-oxidase activities [10]. The growth of pea shoots is under the biphasic control of auxin. The intial cell expansion was a true effect (Phase I) and was associated with the decrease in peroxidase activity. The second phase was regulated by the auxin-induced ethylene production and involved thickening of cell walls and rise in peroxidase activity. The secondary responses of auxin were mimicked by the exogenous application of ethylene. Thus the effects of auxin and ethylene are clearly distinguishable in pea shoots [14, 15]. In wheat coleoptiles [16] and pea stem segments [17, 18], exogenous application of auxin resulted in the repression of peroxidase activity. Also the addition of auxin to cultures of tobacco pith tissue inhibited two anodic peroxidase isoenzymes [19]. In a few cases the regulation of peroxidase activity by auxin was not affected by ethylene. In tobacco pith tissue, the repression of peroxidase multiple forms by auxin was not mimicked by the application of ethylene [6]. Conversely, in sweet potato roots, peroxidase activity was stimulated by ethylene, while auxin gave no such response [8].

In this communication, we report that the enhancement of peroxidase activity in excised mung bean cotyledons was increased by auxin and inhibited by abscisic acid.

RESULTS AND DISCUSSION

Peroxidase activity in germinating cotyledons

Time course studies revealed a relatively high peroxidase activity in detached germinating cotyledons in comparison to the cotyledons attached to the seedling. Acrylamide gel electrophoretic pattern of peroxidase multiple forms was also different in attached and detached cotyledons (Fig. 1); the excised cotyledons exhibited more bands with relatively high intensity. Thus the enhanced enzyme activity in detached cotyledons compared to that of attached cotyledons could be attributed to qualitative and quantitative differences in peroxidase multiple forms. There was a 22–35 fold increase of peroxidase activity in excised cotyledons 72 hr after germination. This was accompanied by the appearance of six additional multiple forms of peroxidase.

Regulation of peroxidase activity by auxin and abscisic acid

Application of GA_3 (10^{-7} M, 10^{-5} M) or kinetin

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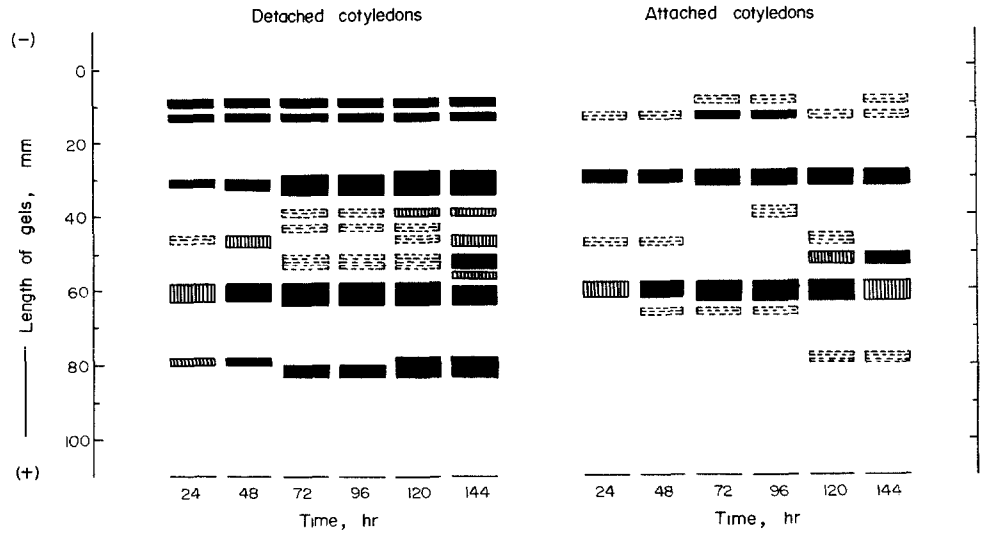

Fig. 1. Diagrammatic representation of peroxidase multiple forms in attached and detached cotyledons at different stages of germination. Extracts of cotyledons were fractionated on acrylamide gels (7.5%) using anionic system (pH 8.3). Multiple forms of peroxidase were developed with the satd soln of benzidene and H_2O_2 (1%). ■ High activity ▥ Medium activity ▤ Low activity.

(10^{-7} M, 10^{-5} M) to the germinating excised cotyledons did not alter the level of peroxidase activity. A significant enhancement of peroxidase activity was witnessed in excised cotyledons germinated in presence of indoleacetic acid (IAA). Increasing concentrations of IAA showed progressive stimulation of peroxidase activity at 72 and 96 hr of germination. Synthetic auxin (2,4-D) proved superior to IAA in the enhancement of peroxidase activity. There was identical extent of stimulation of enzyme activity with both plant growth substances up to 48 hr of germination, whereas at 72 hr stage, 2,4-D elicited a 5-fold increase as against 3 fold increase by IAA. In general, it was observed that IAA could bring about 2–4 fold increase, while in case of 2,4-D there was 4–8 fold enhancement of enzyme activity.

A severe inhibition (87%) of peroxidase activity was observed in excised cotyledons germinated in presence of abscisic acid (ABA, 100 μg/ml). Addition of GA_3 (10^{-5} M, 10^{-4} M) and kinetin (10^{-6} M) failed to counteract the inhibitory effect of ABA (100 μg/ml). However, there was a significant alleviation of ABA-induced inhibition of peroxidase activity by auxin (IAA, 10^{-3} M, 2,4-D 10^{-3} M) (Table 1). This indicated that the stimulation of peroxidase activity in water imbibed cotyledons is regulated by the endogenous pool of auxin.

Table 1. Effect of abscisic acid and auxin on peroxidase activity in germinating excised cotyledons of mung bean

Additions	Enzyme units/ mg protein	Relative activity
Control	3.30	1.00
ABA 100 μg/ml	0.42	0.13
ABA 100 μg/ml + IAA 10^{-3} M	1.80	0.55
ABA 100 μg/ml + 2,4-D 10^{-3} M	2.80	0.85
IAA 10^{-3} M	12.20	3.70
2,4-D 10^{-3} M	22.20	6.73

Excised cotyledons were cultured for 72 hr in continuous light at 25 ± 2°. Abscisic acid and auxin were present throughout the period of germination. Peroxidase activity was measured in *crude extract*.

Addition of auxin (IAA, 10^{-4} M) either during homogenization of cotyledons, or its *in vitro* addition to *crude extract* failed to enhance peroxidase activity. This excluded the effector-like action of auxin on the preformed enzyme molecules.

Effect of ethrel on peroxidase activity

Several metabolic response elicited by supraoptimal concentration of auxin are brought about by the enhanced release of ethylene gas [20, 21]. In all such instances, the exogenous application of ethylene mimicked the response produced by auxin [22, 23]. It was, therefore, considered desirable to ascertain if the auxin-stimulated peroxidase activity in excised cotyledons of mung bean was an ethylene-regulated phenomenon. For this purpose, a wide range of ethrel concentrations (E, 10^{-7} M–10^{-2} M) were tested on germinating cotyledons, but unlike auxin, ethrel proved ineffective in triggering peroxidase activity over the control. Also agents like dichlorophenol (10^{-3} M) and caffeic acid (10^{-3} M) which inhibit auxin-induced ethylene evolution [13], failed to decrease peroxidase activity in auxin-treated mung bean cotyledons. This excluded the involvement of ethylene in the stimulation of enzyme activity. Other workers have also reported that auxin-induced repression of peroxidase was not mimicked by the exogenous application of ethylene [6, 21]. In decapitated pea seedlings, auxin treatment promoted cellulase and cellobiase activities. However, application of ethylene failed to increase the activity of these enzymes, instead the gas inhibited (36%) auxin-induced cellulase activity [22].

Inhibition of rise in peroxidase activity by actinomycin D and cycloheximide

Peroxidase activity was effectively inhibited by act D (50 μg/ml) both in controls and in auxin-treated excised

Table 2. Effect of actinomycin D on control and auxin stimulated peroxidase activity in germinating excised cotyledons of mung bean

Additions	Enzyme units/ mg protein	Relative activity
Control	4.30	1.00
Act D 50 μg/ml	0.70	0.16
IAA 10^{-4} M	7.20	1.68
IAA 10^{-4} M + Act D 50 μg/ml	0.94	0.22
IAA 10^{-3} M	13.70	3.19
IAA 10^{-3} M + Act D 50 μg/ml	1.00	0.23

Excised cotyledons were cultured for 72 hr in continuous light at 25 ± 2°. Act D and auxin were added simultaneously at the beginning of incubation. Peroxidase activity was measured in *crude extract*.

cotyledons (Table 2). Similar inhibition was observed with cycloheximide (CHI, 5 μg/ml). Thus the inhibitor studies indicated the pre-requirement of RNA and protein synthesis for the enhancement of peroxidase activity in excised cotyledons. Inhibition of peroxidase activity by act D and CHI has also been reported in sweet potato root discs [23], tobacco pith tissue [21, 24], lentil axis and pea stem segments [18].

EXPERIMENTAL

Germination of cotyledons. Seeds of mung bean (*Phaseolus aureus* var. *Pusa baisakhi*) were soaked in sterile H_2O for 10 hr at 16° and employed for dissecting out the cotyledons. The excised cotyledons were surface sterilized with $HgCl_2$ soln (0.1%) for 3 min and rinsed thoroughly with sterile H_2O. The cotyledons were germinated under aseptic conditions on sterile H_2O containing chloramphenicol (50 μg/ml). The Petri plates containing cotyledons were maintained under continuous fluorescent light at 25 ± 2°. The effect of different plant growth substances (GA_3, kinetin, auxin, abscisic acid, ethrel) was tested on peroxidase activity in germinating cotyledons. The presoaked intact seeds were also germinated and served as a source of attached cotyledons.

Enzyme extraction. The cotyledons were grinded in Pi buffer (0.05 M, pH 6.6) with white sand as an abrasive agent. The homogenate was centrifuged in Sorvall at 16000 *g* for 20 min in cold (4°). The supernatant fraction (*crude extract*) was employed for measuring peroxidase activity.

Assay of peroxidase activity. Slightly modified procedure of ref. [25] was adopted for the assay of peroxidase activity. The assay mixture comprised of *o*-dininsidine (2.4 μmol), H_2O_2 (20 μmol), *crude extract* (0·05–0·5 mg protein) and Pi buffer (0.05 M, pH 6.6) to make the vol. up to 4·0 ml. Omission of H_2O_2 from the incubation mixture served as blank. A unit of enzyme activity represents a change in *A* of 0.001 at 430 nm/3 min in an incubation mixture of 4·0 ml. The enzyme units are expressed on mg protein basis. The protein was determined in *crude extracts* by the procedure of ref. [26].

Fractionation of peroxidase multiple forms on acrylamide gels. Modified procedure of ref. [27] was followed for the fractionation of *crude extract* on polyacrylamide gels using anionic system. The spacer gel was replaced by sucrose soln (5%). *Crude extract* (100–200 μg protein in an aliquot of 0.1 ml) containing sucrose (10%) was gently layered on each gel column. Bromophenol blue was used as a tracking dye and was mixed with Tris-glycine buffer (pH 8.3). The multiple forms of peroxidase were developed by the procedure of ref. [28]. The gels were rinsed with Pi buffer (0.05 M, pH 5.1) and then incubated for 3 min at 30° in a freshly prepared satd soln of benzidene (dissolved in 25% HOAc) mixed with equal vol. of H_2O_2 (1%). Thereafter, the gels were rinsed with H_2O and stored in dilute HOAc soln (7%). The position of the activity bands of peroxidase was recorded on graph paper.

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