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IAA OXIDASE AND POLYPHENOL OXIDASE ACTIVITIES OF PEANUT PEROXIDASE ISOZYMES

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Abstract—Four anionic isozymes (A_1, A_2, A_4 and A_5) from peanut cells in suspension medium possessed IAA oxidase and polyphenol oxidase activities. The specific activities of each of the enzymes differed among the 4 isozymes. The pH optima established in these assays for peroxidase was acidic, for IAA oxidase neutral and for polyphenol oxidase alkaline. All 4 isozymes had different K_m and V_{max} for the enzyme activities of peroxidase and polyphenol oxidase. The sigmoid kinetics from the IAA oxidase assays for the isozymes probably indicates an allosteric nature.

INTRODUCTION

Peroxidase (donor: H_2O_2, oxidoreductase; EC 1.11.1.7) is an enzyme defined by its ability to oxidize a wide variety of hydrogen donors in the presence of H_2O_2 [1]. In addition, peroxidase is capable of oxidizing several compounds in the absence of H_2O_2 e.g. chalcones [2, 3], tyrosine and tyramine [4], $NADH_2$ and $NADPH_2$ [5] etradiole, thiols [6], pyridoxal related compounds [7] and indole acetic acid (IAA) [8].

The curiosity of several investigators has been centered around the unique nature of the peroxidase molecule because of its probable relationship with IAA oxidase and polyphenol oxidase (PPO) [9–11]. It could be that the physiological significance of peroxidase in plants may be explained by its relationship with IAA oxidase and PPO.

Horseradish peroxidase catalyzes the oxidation of IAA [8, 12]. However, whether the oxidation of IAA by peroxidase is a general phenomenon is not well understood. Three hypotheses exist regarding the relationship between peroxidase and IAA oxidase: (a) peroxidase and IAA oxidase are different enzymes [13, 14], (b) peroxidase and IAA oxidase activities are associated with the same protein molecule but have different active sites [15–17], (c) only a few of several existing peroxidase isozymes possess IAA oxidase activity [18].

An interrelationship among peroxidase and PPO has also been speculated [10, 19] but it has been disputed by some workers [20, 21]. Thus, an interrelationship among peroxidase, IAA oxidase and PPO is still obscure. In a previous report, based on the similar elution profile from ion-exchange columns and zymograms, we proposed an interrelationship among peroxidase, IAA oxidase and PPO [22]. In this report such an interrelationship among the 3 enzymes has been further explored at the isozymal level. In addition, based on such an interrelationship, a hypothesis has been proposed regarding the physiological significance of peroxidase in plants.

RESULTS AND DISCUSSION

Of the 7 known peroxidase isozymes. found in the culture medium of peanut cell suspension, 4 (A_1, A_2, A_4 and A_5) were examined for peroxidase, IAA oxidase and PPO activities. All 4 isozymes possessed the 3 enzyme activities but showed differences in their sp. act. Using guaiacol as a H-donor during peroxidase assay, A_5 showed the highest sp. act. followed by A_4, A_2 and A_1 (Table 1). With pyrogallol as a H-donor, A_4 revealed the highest sp. act. followed by A_5, A_2 and A_1. Similarly, A_4 had the highest sp. act. with *o*-dianisidine as a H-donor followed by A_5, A_2 and A_1. In order to determine if any correlation exists among the sp. act. of the isozymes during peroxidase activity with the 3 H-donors, the ratios of their sp. act. were calculated. The ratio was around unity for A_2, but the other isozymes did not show any meaningful relationship.

All 4 isozymes also showed differences in their sp. act. during PPO activity (Table 2). They all utilized L-DOPA and DL-DOPA as a substrate, but not D-DOPA. This suggests the stereospecific nature of the active site for PPO activity of isozymes.

Since PPO activity can be subdivided into catecholase and cresolase activities, the 4 isozymes were examined for these activities. All 4 possessed both activities but the ratio among their cresolase and catecholase sp. act. were different (Table 3).

Table 1. Sp. act. of isozymes with three H-donors during peroxidase activity

Substrate	Specific activity of isozymes* (e.u. per mg of protein)			
	A1	A2	A4	A5
Guaiacol	0.066	0.16	0.493	0.78
Pyrogallol	0.056	0.148	0.226	0.173
o-Dianisidine	0.098	0.247	0.382	0.35

* Values are the mean of 3 replicates.

Table 2. Sp. act. of isozymes for polyphenol oxidase activity

Substrates	Specific activity of isozymes*			
	A1	A2	A4	A5
D-DOPA	0	0	0	0
L-DOPA	0.02	0.02	0.02	0.1
DL-DOPA	0.04	0.05	0.03	0.16

* Values are the mean of 3 replicates.

Table 3. Specific activity of isozymes during creolase and catecholase activities

Isozymes	Catcholase*	Cresolase*	Catechlase /cresolase
A1	2520	490	5.14
A2	5600	700	8.0
A4	1000	3000	0.33
A5	1207	845	1.42

* Sp. act. is defined as μl of O_2 consumed per mg of protein. The values reported are the mean of 3 replicates.

During IAA oxidase assay, with IAA as the substrate and DCP (2,4-dichlorophenol) and $MnCl_2$ as co-factors, the isozymes again showed differential sp. act. Furthermore, if the cofactors were omitted during the assay, a loss in sp. act. was noted (Table 4). This suggested that the cofactors were essential for the IAA oxidase activity of isozymes.

The differences in sp. act. of isozymes represent differences in their catalytic capacity. Differences in sp. act. of isozymes isolated from various other plant tissues, during peroxidative [23, 24] and oxidative reaction i.e. IAA oxidation [25] and DL-DOPA oxidation [26], have been reported.

During peroxidative reaction, all 4 isozymes possessed different pH optima with either guaiacol, pyrogallol or *o*-dianisidine as a H-donor (Table 5). With guaiacol as a H-donor, pH optima were between 4.4 and 6.4. With the same H-donor, the pH optima or two tomato peroxidase isozymes were 5 and 8 [27] whereas it was 6 and 7 for two isozymes isolated from culture medium of kidney bean cell suspension [28]. Thus, with guaiacol as a H-donor a wide range pH optima were noticed for peroxidase isozymes from various sources.

With pyrogallol as a H-donor, the optimal pH was 6 for A_1 and A_4 whereas it was 8 for A_2 and A_5 (Table 5). Using the same H-donor, Evans [27] found that the two peroxidase isozymes from dwarf tomato plants had pH optima of 8. All 4 isozymes possessed · pH optima between 4.4 and 6.0 with *o*-dianisidine as a H-donor (Table 5). Kay *et al.* [29] have also noted an acidic pH optimum for horseradish peroxidase isozymes using the same compound.

Peanut peroxidase isozymes were exceptional in having an optimal pH near neutrality for IAA oxidase activity. The peroxidase isozymes from other sources have generally acidic pH optima during IAA oxidase activity [28, 30]. Similar to the peanut peroxidase isozymes, the pH optima for PPO activity of peroxidase isozymes from various sources was 8 [10, 31]. Thus, all the 3 enzyme activities were associated with the 4 peanut peroxidase isozymes but with different pH optima i.e. acidic for peroxidase, neutral for IAA oxidase and alkaline for PPO. The differences in pH optima of the peanut peroxidase isozymes may represent differences in their active sites and in their substrate binding sites.

Differences in K_m and V_{max} values of each isozyme were also observed during their peroxidative and oxidative reactions (Table 6). With an increasing concentration of guaiacol, a typical Michaelis–Menten saturation curve (rectangular hyperbola) was observed for A_1 and A_4. However substrate inhibition was observed for A_2 and A_5. The highest V_{max} and K_m was observed for A_5 (Table 6). With increasing concentrations of pyrogallol as a H-donor, the rectangular hyperbola curves were observed for A_1, A_4 and A_5, whereas A_2 showed a substrate inhibition. All 4 isozymes differed in their K_m and V_{max} (Table 6). With *o*-dianisidine as a H-donor, A_2 and A_5 showed a typical Michaelis–Menten curve whereas A_1 and A_4 revealed a substrate inhibition. A_4 had the highest V_{max} followed by A_5, A_1 and A_2. The highest K_m

Table 4. Sp. act. of isozymes for IAA oxidase activity

Substrate	Cofactors		Percent specific activity* remaining without cofactor			
	$MnCl_2$	2,4-DCP	A1	A2	A4	A5
IAA	+	−	100	64	100	37
IAA	−	+	92.8	72	70	37
IAA	−	−	18.9	72	26	26

* Sp. act. is defined as μg IAA oxidized per mg of protein. The values are the mean of 3 replicates.

Table 5. pH optima of isozymes during their peroxidase, IAA oxidase and PPO activities

Isozyme	Peroxidase			IAA oxidase	Polyphenol oxidase
	Guaiacol	Pyrogallol	*o*-Dianisidine		
A1	5.2	6	6	7.6	7.6
A2	5.6	8	6.4	7.2	8
A4	4.4	6.4	4.4	7.6	8
A5	6.4	8	5.6	7.6	8
Mixture*	5.2	6 and 8	4 and 5.6	7.6	7.6

* Mixture contained all 7 peroxidase isozymes found in peanut cell culture medium.

Table 6. K_m and V_{max} of isozymes during peroxidative reaction with guaiacol, pyrogallol or *o*-dianisidine as an H-donor and during oxidative reaction with DL-DOPA as the substrate

Isozymes	Peroxidase (K_m (mM) Guaiacol	Pyrogallol	*o*-Dianisidine	V_{max} Guaiacol	Pyrogallol	(ΔO.D./min/ml) *o*-dianisidine	Polyphenol oxidase K_m DL-DOPA (mM)	V_{max} DL-DOPA ΔA/min/ml
A1	58.82	1.25	20	1.54	0.5	0.13	100	0.01
A2	35.6	9.5	18.2	2.5	2.8	0.11	20	0.5
A4	34.48	1.43	10	1.11	1.18	0.5	20	0.34
A5	125	6.25	9.1	3.33	1.33	0.41	15.4	0.016

was observed for A_1 followed by A_2, A_4 and A_5. During PPO activity, all 4 isozymes revealed a Michaelis–Menten saturation curve. The highest K_m was observed for A_1 followed by A_2, A_4 and A_5. The highest V_{max} was observed for A_2 followed by A_4, A_5 and A_1.

When an increasing concentration of IAA was used during IAA oxidase assay, a sigmoid curve rather than a typical Michaelis–Menten curve was observed for each isozyme (Fig. 1). The fundamental observation on which most enzymes were regarded as regulatory or allosteric in nature is their sigmoid kinetics. Thus, the sigmoid curve of peanut peroxidase isozymes may represent their regulatory nature during IAA oxidase activity. Three major classes of regulatory enzymes are now recognized: (a) Homotropic—substrate also acts as a modulator, (b) Heterotropic—compounds other than the substrate acts as a modulator, (c) Homotropic–heterotropic—both substrate and another compound act as a modulator. Peanut peroxidase isozymes are thus probably homotropic in nature. A report has been published indicating the allosteric nature of IAA oxidase isozymes from cabbage (32].

Examples of a single protein molecule having more than one enzyme activity are scarce in literature, but not altogether absent. Two threonine-sensitive activities from *E. coli*, β-aspartokinase and homoserine dehydrogenase are reported to be associated with a single protein molecule [33]. Similarly plant phenolase are also known to have dual enzyme activities i.e. hydroxylation and oxidation of phenolic compounds [34]. Therefore, a protein molecular having peroxidase, IAA oxidase and PPO activities may also be conceivable.

Diphenols and other phenolic compounds are derived in cells via the shikimic acid pathway which utilizes erythrose-4-phosphate (obtained from the phosphogluconate pathway). Phenylalanine, a product of this pathway, is converted to tyrosine and in turn to cinnamic acid. Cinnamic acid is then converted to various phenolic compounds and also to lignin. Furthermore, anthranilic acid, a by-product of the shikimic acid pathway, gives rise to tryptophan which in turn produces IAA. The peroxidase molecule, by virtue of the 3 enzyme activities, could control the shikimic acid pathway favouring either lignin formation, phenolic oxidation or an accumulation of IAA. By oxidizing IAA (formed via the shikimic acid pathway) peroxidase may induce more IAA synthesis because an optimal level of IAA has to be maintained in plant cells. Alternatively, IAA may also act as an effector molecule during allosteric activity of IAA oxidase; therefore as soon as a higher than optimal level of IAA concentration is reached in cells, IAA would enhance IAA oxidase activity and thus maintain an optimal level.

Polyphenol oxidase could be regulatory since its oxidase activity would control the levels of *o*-diphenols [20]. The *o*-diphenols are inhibitory for the IAA oxidase activity. Therefore, by oxidizing the phenols through the PPO activity of IAA oxidase molecules, the IAA oxidase activity of cells may be kept functional. Alternatively, the phenolic compounds may also be utilized

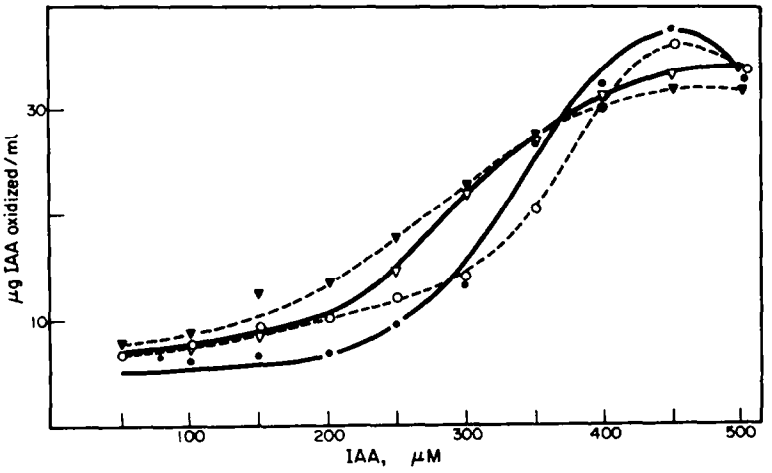

Fig. 1. Effect of IAA concentration on IAA oxidase activity isozymes. ●—●—● isozyme A_1, ▼—▼—▼ isozyme A_2, ○—○—○ isozyme A_4 and ▽—▽—▽ isozyme A_5.

as H-donors during peroxidase activity or by their polymerization to lignin precursors e.g. eugenol, syringaldehyde, coniferaldehyde. Thus an association of peroxidase, IAA oxidase and PPO activities to a single protein molecule would be highly beneficial for plants.

EXPERIMENTAL

The 7 peroxidase isozymes (anionic A_1, A_2, A_3, A_4 and A_5 and cationic isozymes C_1 and C_2) were purified and separated as described before [22]. However, an additional step of re-precipitation of isozymes by $NH_4(HNu)_2SO_4$, after Me_2CO precipitation was included. Four anionic isozymes A_1, A_2, A_4 and A_5 were used during the present studies.

Optimal pH determination of isozymes. The following buffers (0.1 M) were used; acetate pH 3.6–5.6, Pi pH 6.2–7.8 and borate pH 7.8–9.2. Three hydrogen donors (guaiacol, pyrogallol and *o*-dianisidine) were employed during the pH optima determination. The reaction mixture contained 0.5 ml of an isozyme prepn, 2 ml of buffer of desired pH, 1 ml of 0.1 M of a H-donor and 0.5 ml of 0.3% H_2O_2. The peroxidase, PPO and IAA oxidase assay were carried out as described before [22].

The specific activity determination of isozymes for peroxidase activity was carried out with 3 H-donors, guaiacol, pyrogallol and *o*-dianisidine. The peroxidase, IAA oxidase and PPO assay were done as described in ref. [22]. During PPO assay, D-DOPA L-DOPA and DL-DOPA were used as substrates. The cresolase and catecholase assay were carried out using the method of ref. [35].

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