PEROXIDASE ACTIVITY AND LIGNIFICATION IN THE POD MEMBRANE OF *PISUM SATIVUM* L.*

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Abstract

An investigation of four mutant genotypes of *P. sativum* has shown an inverse relationship between peroxidase activity and lignification of the pod membrane. From developmental studies, it appears that the main role of peroxidase is in cellular growth and differentiation. In this context it is suggested that peroxidase may predispose cells to lignification without being directly involved in the lignification process *per se*.

Introduction

On the basis of histochemical studies, Freudenberg *et al.* (1952) proposed that peroxidase was involved in lignification *in vivo* in spruce. This view found support from the work of Jensen (1955), and Siegel (1956, 1957) later demonstrated that peroxidase would effect an *in vitro* oxidation of eugenol to a lignin-like substance. More recent studies by Van Fleet (1959), Wardrop and Bland (1959), and Koblitz and Koblitz (1966) argue against the direct involvement of peroxidase in the lignification process. In particular these workers have shown that maximum peroxidase activity occurs in prolignifying tissues, and that activity declines after lignification of the primary wall has commenced. Further, De Jong (1967) claims never to have observed a positive peroxidase reaction in xylem.

However, it is evident from studies such as those of Lipetz and Garro (1965) and Parish and Miller (1969), that there may be some relationship, even though an indirect one, between peroxidase and lignification. These workers have demonstrated the presence of both soluble and wall-bound peroxidases in plant tissues, and have shown further that treatments which cause leaching of wall-bound peroxidases also reduce lignification.

Recent work has shown that the "peroxidase" of most plant tissues consists of a number of isoenzymes, or components with peroxidase activity (e.g. Mills and Crowden 1968; Alvarez and King 1969). These components are not usually differentiated in histochemical tests. This raises the possibility that there may be a division of function of peroxidases *in vivo*, and that only specific components of the peroxidase complex may be associated with lignification. In this context we have investigated peroxidase activity in some mutant plants of the garden pea, *Pisum sativum* L. In this species, lignification of the inner pod membrane is determined by two Mendelian genes, *P* and *V*. Normal pods, *PV*, contain a broad inner membrane

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several cells thick, which lignifies during maturation of the pod. Mutations in either of the genes *P* and *V* effect the formation and subsequent lignification of this membrane. *Pv* pods form a complete membrane, which lignifies in "strings". In *pV* pods, the membrane is reduced in thickness over most of the pod, except near the main vein, where lignification occurs. The membrane of pods of genotype *pv* is further reducde in thickness, more so than in *pV*, and no lignification occurs.

In this investigation we have studied peroxidase localization and activity, and the progress of membrane lignification during pod development and maturation in all four genotypes.

Materials and Methods

The mutant plants used in this investigation were obtained from the collection of Dr. I. C. Murfet, Botany Department, University of Tasmania. The following lines were used: 22, 24, genotype *PV*; 13, 39, genotype *Pv*; 1, 8, 15, genotype *pV*; 2, genotype *pv*. In addition, some investigations were made using line 1451 obtained from Dr. S. Blixt, Plant Breeding Institution, Weibullsholm, Landskrona, Sweden. This line has been genotyped *pv*. It produces a membrane several cells thick (as in *PV* and *Pv* genotypes), but the membrane does not lignify.

Plants were grown in containers having 1 : 1 vermiculite–dolerite chips (0·5-cm mesh) solid medium. They were watered daily and normal Hoagland's nutrient solution was applied weekly. Pods were harvested at intervals throughout their development and examined fresh. Generally pods were studied at six stages of development;

(1) very young pod, the flower having just withered;

(2) pod partially extended, up to 2 cm long;

(3) pod fully extended, but expansion of ovules not commenced;

(4) pod with ovules partially expanded;

(5) nearly mature pod;

(6) mature pod.

Histochemical Studies.—Cross-sections of pods were cut by hand. Peroxidase distribution in the tissues was determined using the method of Alvarez and King (1969). Lignin was stained by treating sections for several minutes in a saturated solution of phloroglucinol in 20% HCl at 20°C.

Peroxidase Activity in Pod Membranes.—The inner membrane could be readily torn off older, lignifying pods, but with young pods and sugar pods the membrane was best prepared by tedious and careful scraping with a razor-blade. The material so obtained was ground with fine sand in phosphate buffer (pH 7·0, 0·02M), centrifuged at 20,000 *g* for 10 min, and the supernatant used to assay soluble peroxidases, and for electrophoresis. To investigate membrane-bound peroxidase, the tissue residue was first washed repeatedly with an excess of phosphate buffer and the washings discarded.

Peroxidase (both soluble and bound), was assayed by the method of Jermyn and Thomas (1954). Protein was determined by the method of Lowry *et al.* (1961). Electrophoretic examination of peroxidase enzymes was carried out using the method of Mills and Crowden (1968).

Results

(i) *Histochemical Studies.*—Peroxidase was shown to be present in pod membranes of all four genotypes. In stages 1 and 2 of *PV* membranes, the peroxidase stain was uniform, but not very intense, and somewhat less than that of the neighbouring cortical cells of the pod. Lignification of the membrane commenced only after the pods were fully extended. The inner layer of membrane cells became elongated, then lignified, followed in sequence by the next layer of cells, and so on. The intensity of

peroxidase staining was seen to increase prior to lignification, especially in the inner layers of the membrane, but decreased after there was significant deposition of lignin.

There was no peroxidase activity in fully lignified cells. Except in very young cells, the crystals of benzidine blue were always closely associated with the cell wall, but were never actually seen inside the wall. The peroxidase activity in membranes of *Pv* pods was similar to *PV*. However, *Pv* membranes, although uniformly thick only lignified in certain regions forming lignin "strings". In the prelignified *Pv* membrane (stage 2) regions containing somewhat elongated cells appeared to stain more strongly for peroxidase than did the surrounding cells. These darker-staining cells subsequently lignified. Lignification of membranes of *pV* pods appeared to follow a similar sequence, but was restricted to a region near the main vein. Despite the localized occurrence of lignification, all cells in the membrane stained positively for peroxidase. Considerable peroxidase activity was also observed in the cells of the non-lignifying membrane of genotype *pv* and the intensity of peroxidase staining in these cells appeared to increase continuously as the pods matured.

TABLE 1

PEROXIDASE ACTIVITY IN POD MEMBRANES OF MUTANT PEA LINES

Activity estimated as absorbance at 400 nm per milligram protein

Genotype	Line	Activity of membrane tissue at following stages of pod development:						Activity of pod tissue
		1	2	3	4	5	6	
				(*a*) Soluble peroxidase				
PV	22		9·01		19·31		13·48	3·96
	24		6·15		7·31		3·73	2·58
Pv	14		5·89		7·44		8·19	3·80
	39		—		7·14		8·12	4·16
pV	1		7·98		5·69		8·70	8·20
	15		6·02		6·80		—	4·18
pv	2		3·36		6·78		5·87	3·19
	1451		6·47		7·71		10·46	5·74
				(*b*) Wall-bound peroxidase				
PV	22	17·70	36·00	48·50	42·40	15·90	21·60	
	24				29·20		9·71	
Pv	14		16·40	43·50		24·50		
	39		14·40		27·80	23·80	19·08	
pV	1			19·80	26·10	22·20		
	15		13·36	17·83		22·80	17·23	
pv	2		18·58			19·10	37·00	

(ii) *Peroxidase Assays.*—The results are shown in Table 1. Except for line 1 (*pV*) the peroxidase activities of the membranes were considerably higher than those of the corresponding pod tissues. Soluble peroxidase activity from *PV* membranes rose initially then fell as the membrane matured, but in other genotypes, peroxidase

activity generally increased with aging of the tissue. Two exceptions to this were observed, viz: in stage 4 membrane of line 1 (*pV*) and stage 6 membrane of line 2 (*pv*).

The results for wall-bound peroxidases showed that in all cases [except line 2 (*pv*), where the activity continued to increase with age] there was an initial rise followed by a decline in peroxidase activity during development, though the maximum activity occurred at different stages for different genotypes. There was insufficient membrane to carry out concurrent histochemical investigation of all stages, but in those cases (e.g. line 22) where this was possible, it was evident that the activity of bound peroxidase was maximum at the time of commencement of lignification. Thereafter activity fell dramatically as lignification progressed.

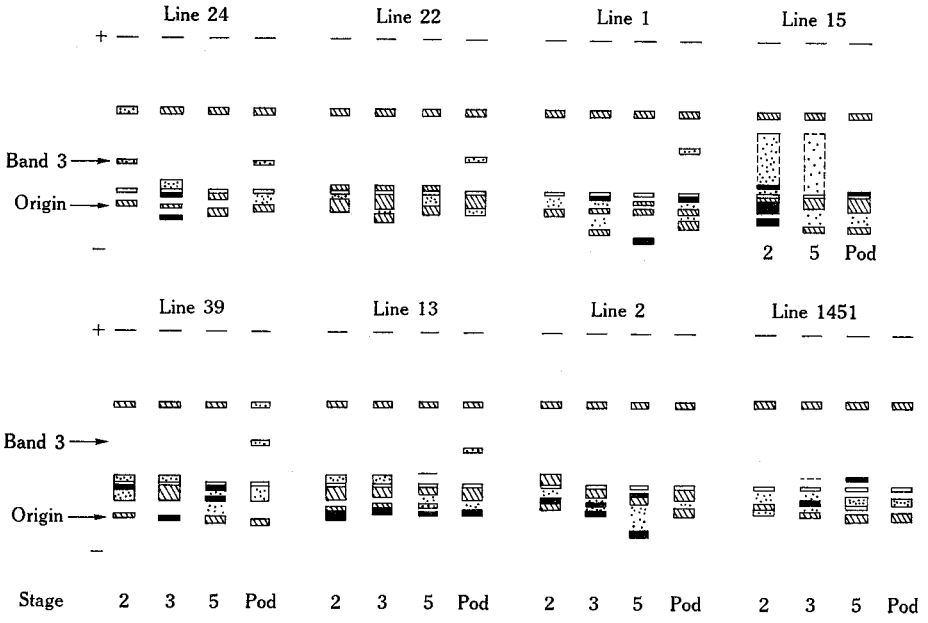

Fig. 1.—Interpretative drawings of acrylamide-gel electrophoretograms of the soluble peroxidases in extracts from pod membranes of mutant varieties of *P. sativum*. Developmental stages 2, 3, and 5 and pod tissue are represented for each genotype except for line 15 which is for stages 2 and 5 and pod tissue. Band 3 is labelled.

(iii) *Electrophoresis of Soluble Peroxidases.*—The qualitative variation seen on electrophoretograms generally was not consistent with differences in genotype (Fig. 1). However, each genotype showed a varying complement and activity of peroxidases during development, and in general there was good correlation between the intensities of band-staining on the electrophoretograms and assays of soluble peroxidases (Table 1). Band 3, which was not seen in membrane extracts, was restricted to pod tissues of genotypes *PV*, *Pv*, and *pV*, where it could be used as a diagnosis that the membrane preparations were not contaminated by pod cells. We were unable to obtain suitably concentrated preparations of wall-bound peroxidases (by leaching with Ca^{2+}) for electrophoretic investigation.

Discussion

From the results obtained in this investigation, there does not appear to be any direct involvement of a peroxidase enzyme in the lignification process. In agreement with other workers, we have observed that peroxidase reaches a maximum activity prior to the commencement of lignification. In *PV* membranes (lines 22 and 24), which lignify uniformly, the peroxidase activity, both soluble and bound, declines immediately lignification commences. However, in those genotypes which do not lignify uniformly, *Pv* (lines 13 and 39) and *pV* (lines 1, 8, and 15), the soluble peroxidase component continues to increase in activity throughout pod development, whilst the wall-bound component shows a progressive rise and fall in inverse correlation to the onset of lignification. It is most probable that the soluble peroxidases which continue to increase in amount are contained in the non-lignifying cells of these membranes. In the non-lignifying genotype, *pv* (line 2) both soluble and bound peroxidases increase throughout the whole period of pod maturation. The decline in peroxidase activity in *PV* membranes (and in lignifying cells of *Pv* and *pV* types), is probably a simple reflection of the reduced metabolic activity of these cells leading to the eventual death of the protoplasts, as the deposition of lignin cuts off their food supply.

At this stage speculation on the specific functions or the degree of interaction of the soluble and wall-bound peroxidases is difficult. It appears that the principle role of peroxidases in the pod membranes is in relation to the normal growth processes of the cells. Other workers, e.g. Galston and Dalberg (1964), and Parish (1969), have observed increasing peroxidase activity in maturing plant tissues, and Van Fleet (1959) has suggested that peroxidase has significance in respect of differentiation as well as growth processes in cells. Peroxidase may well predispose cells to lignification without being directly involved in the lignification process *per se*. Thus, in *Pv* and *pV* membranes, cells in localized regions wherein lignification subsequently occurred, i.e. those cells which had become noticeably elongated, had a more intense peroxidase-staining reaction than did the neighbouring non-differentiating cells.

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