J Plant Growth Regul (1986) 5:17–27

Journal of
Plant
Growth
Regulation

Role of Peroxidase and Esterase Activities During Cotton Fiber Development

Vrinda S. Thaker, Sant Saroop, Pankaj P. Vaishnav, and Yash Dev Singh

Department of Biosciences, Saurashtra University, Rajkot-360 005, India

Received July 17, 1985; accepted November 18, 1985

Abstract. Cytoplasmic and salt-extracted wall peroxidase and nonspecific esterase activities along with growth analysis were investigated during the entire period of cotton fiber development. Both the peroxidase fractions, when assayed with chlorogenic and ferulic acids as substrates, recorded low levels during the fiber elongation phase, and a close relationship between cessation of elongation growth and increase in peroxidase activity was discernible. Nonspecific esterase activity in both cytoplasmic and salt-extracted fractions, on the other hand, showed higher activity during the elongation phase, whereas during the secondary thickening phase it decreased. The role of cytoplasmic peroxidase in IAA oxidation is discussed. It is suggested that esterases and peroxidases associated with wall fractions may well be involved in turnover of phenolic acids that are cross-linked to wall polysaccharides.

Phenols associated with cell wall are proposed to play an important role in cell expansion (Fry 1982a, Taiz 1984). Occurrence of polysaccharide esters of ferulic acid, not only in lignified cell walls but also in cell walls of many growing tissues which fail to show the classic lignin-histochemical test, has been reported (Harris and Hartley 1976, Smith and Hartley 1983). Such phenols can undergo oxidative coupling reaction, catalyzed by extracellular peroxidase, to produce dimers which may cross-link the cell walls and hence restrict expansion growth (Fry 1979). Diferulic acid has been identified in cell walls (Hartley and Jones 1976, Markwalder and Neukom 1976). Additionally, Fry (1982b) discovered a new phenolic amino acid—isodityrosine—in plant cell walls which is an oxidatively coupled dimer of tyrosine in which two tyrosine units are linked by a diphenyl ether bridge. Cooper and Varner (1983) also reported its presence in carrot cell walls and showed that a variety of reagents that block the formation of isodityrosine by inhibiting the peroxidase reaction also inhibit the incorporation of a salt-soluble, hydroxyproline-rich protein—"extensin"—into a tightly bound fraction of the wall.

Central to both isodityrosine and diferulate cross-link models of wall extensibility are the presence and activity of wall-bound peroxidase (Taiz 1984). Although there are many studies on peroxidase activity, a negative correlation between cytoplasmic peroxidase activity and growth rate has often been reported (see references in Lamport and Catt 1981; Fry 1979, 1982a), reports on wall-bound peroxidase activity are few (Ridge and Osborne 1970, Gardiner and Cleland 1974, Fielding and Hall 1978, Gaspar et al. 1983), and doubts have been expressed regarding the *in vivo* significance of these results. This is because the natural substrates of this enzyme are still unknown and in many studies artificial substrates have been utilized. The overlapping specificity of peroxidase for a large number of substrates, the differing ability of individual isoenzymes to utilize them, and the use of artificial hydrogen donors as substrates obscure the physiological significance of this enzyme (Gibson and Liu 1978).

Further, a corollary to the diferulate cross-link model is that extracellular esterases may play a vital role in cleaving the ester links, since ferulic acid is ester-linked to plant cell walls (Smith and O'Brien 1979). Therefore, in the present work, we have studied the changes in cytoplasmic and salt-extracted wall peroxidase and esterase activities during the entire period of cotton fiber development. Ferulic and chlorogenic acids were used as substrates for peroxidase assay.

Cotton fiber is a unicelled, epidermal trichome on the seed coat. It starts initiation on the day of anthesis, and the entire period of its development can be divided into four distinct phases—initiation, elongation, secondary thickening, and maturation (Jasdanwala et al. 1977; Naithani et al. 1981, 1982; Rama Rao et al. 1982a). Being a single cell with distinct synchronous growth phase, cotton fiber is a good system to understand the mechanisms regulating extension growth.

Materials and Methods

Seeds of cotton (*Gossypium hirsutum* CV. SRT-1) were grown under field conditions. The cultural practices including irrigation, application of fertilizer and insecticides, etc. were conducted to maximize lint yield. On the day of anthesis, each individual flower was tagged and the bolls were harvested for analyses after the desired periods.

Growth Analysis

Fiber length was determined by the method of Gipson and Ray (1969). A locule from a boll was placed in boiling water to allow the seeds to separate from each other, and each seed was placed on the convex side of a watchglass. The fibers were streamed out with a jet of water. The length of the fiber was measured from the rounded side of the seed adjacent to the chalazal end. Seeds from three locules were used from three bolls, and mean fiber length was calculated. The fibers were removed from the seed with a scalpel without removing the seed coat, and the dry weight was determined after drying in an oven at 80°C

for 2 days. Each result represents an average of three bolls harvested randomly at a given age.

Preparation of Enzyme Extract

Freshly harvested bolls were opened with a scalpel, and fibers separated from the ovules and stored in a freezer. During the early stages it was difficult to separate the fiber from the ovules, so young ovules were taken for analysis; at d 9 (for esterase) and d 12 (for peroxidase) after anthesis and subsequently, the fibers were used. The frozen material was crushed in a cooled mortar with 0.2 M Na borate buffer (pH 7.6), as suggested by King (1971), to yield maximum proteins. The extract was centrifuged at 1,500*g* for 10 min to obtain the wall fraction, and the supernatant was clarified by centrifuging at 15,000*g* for 10 min. The clarified supernatant was mixed with two parts of chilled acetone at 0°C to precipitate soluble proteins. The precipitate was separated by centrifuging in cold (0–3°C) at 15,000*g* for 10 min and stored at −5°C. The acetone-precipitated proteins were dissolved in Na phosphate buffer (0.02 M, pH 6.4) and used as the enzyme source for peroxidase and esterase activities.

Preparation of Ionically Wall-Bound Enzyme

The wall fraction separated after borate extraction was washed several times with Na phosphate buffer (0.02 M, pH 6.4) by resuspending the wall material, and was centrifuged until no peroxidase activity was recorded in the supernatant. The residue was then extracted in 1 M NaCl for 1 h with constant shaking to release ionically wall bound enzyme and was centrifuged at 15,000*g* for 10 min. The supernatant obtained was used as the source of salt-extracted enzyme. In a control experiment, cell walls were obtained from fibers of different ages, after homogenizing with distilled water. Addition of 0.2 M Na borate buffer (pH 7.6) in cold (0–4°C) did not elute cell wall peroxidases.

Peroxidase Assay

Continuous spectrophotometric assay employed for peroxidase activity with ferulic and chlorogenic acids was similar to that described by Gibson and Liu (1978). The reaction mixture consisted of 12 mM Na phosphate buffer (pH 6.4), 4 mM ferulic acid or chlorogenic acid, 1 mM H_2O_2, and the enzyme. The reaction was initiated by adding H_2O_2, and the increase in absorbance was monitored at 400 nm. The activity is expressed as $\Delta A_{400}\ h^{-1}$ (mg protein)$^{-1}$.

Esterase Assay

Nonspecific esterases were estimated as per Pantelouris (1968). The assay mixture consisted of 62.5 mM TRIS-citrate buffer (pH 7.4), 1.34 mM α-naphthyl acetate, and the enzyme. After 30 min of incubation at 30°C, 1 ml of

80% (w/v) trichloroacetic acid (TCA) and 1 ml of Fast Blue B salt (2 mg/ml DW) were added, and the contents were shaken vigorously. The pink-colored product was extracted with ethyl acetate, and its absorbance was measured at 570 nm. The enzyme activity is expressed as μg α-naphthol released min^{-1} $(mg\ protein)^{-1}$.

Protein Determination and Statistical Background

The protein content of acetone-precipitated cytoplasmic extract, which was more or less free from phenolic interference, and salt-eluted wall extract was measured by the method of Lowry et al. (1951). The complete biochemical analysis was done in triplicate, from which mean values with standard deviations were calculated.

Results

Growth Analysis

The relationship between boll age in days (X) and fiber length in mm, or fiber dry weight in mg (Y), showed a sigmoidal pattern. They were fitted to a logistic curve with the equation

$$Y = \frac{C}{1 + Ar^x}$$

where $A = 0.31744 \times 10^2$, $C = 0.27168 \times 10^2$, and $r = 0.8344$ when Y = fiber length (mm), and $A = 0.6020 \times 10^2$, $C = 0.83289 \times 10^2$ and $r = 0.9019$ when Y = fiber dry weight (mg).

The best-fit curve for fiber length (Fig. 1a) showed that the elongation starts around d 3 after anthesis and continues up to d 33. No significant increase in fiber length was observed during the subsequent period. The dry weight increases, on the other hand, showed a definite lag phase up to d 15–20, after which it increased exponentially until the boll opened (Fig. 1a).

The rates of fiber elongation and dry weight increase per day were determined by using the derivative equation

$$\frac{dy}{dx} = \frac{-CAr^x \log_e r}{(1 + Ar^x)^2}$$

The derivative plot for fiber elongation (Fig. 1b) showed a gradual increase in the rate, reaching the highest level on d 18. During the subsequent period, it decreased gradually and ceased completely on d 45 postanthesis. A similar trend was observed in the rate of dry matter accumulation. However, the highest rate in this case was achieved on d 39 after anthesis, and significant accumulation continued until the boll opened—i.e., up to d 51 after anthesis.

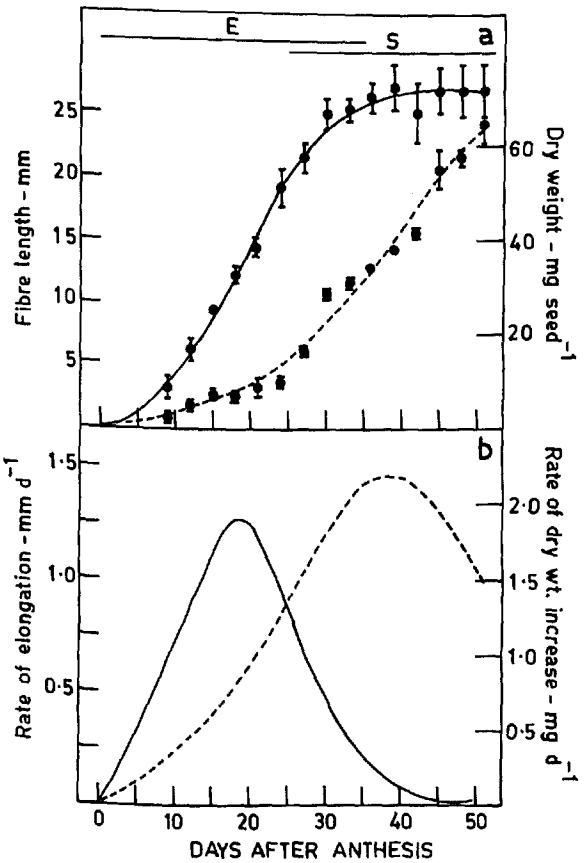

Fig. 1. (a) Best-fit curves for fiber length and dry weight per seed against boll age. (b) The rate curve for fiber elongation and dry weight; fiber length, continuous line; fiber dry weight, broken line. E and S represent the elongation and secondary thickening phases, respectively. Vertical bars represent standard deviation.

Peroxidase Activity

Similar trends were discernible when ferulic acid (Fig. 2) and chlorogenic acid (Fig. 3) were used as hydrogen donors for cytoplasmic or salt-extracted wall peroxidase activity. In unfertilized ovules, a substantial cytoplasmic peroxidase activity was observed (Figs. 2a, 3a). Until d 9 postanthesis, the activity decreased, and a low level was maintained during the entire period of fiber elongation. After 30 days of anthesis, the activity increased rapidly, and high levels were maintained during the secondary thickening phase. Salt-extracted peroxidase activity showed similar trends, except that a rapid increase in the activity was recorded after d 33 of anthesis (Figs. 2b, 3b).

Esterase Activity

When nonspecific esterases were estimated, the bolls had a shorter developmental cycle (opened on d 39) owing to high environmental temperature. In cytoplasmic fraction, considerable nonspecific esterase activity was recorded

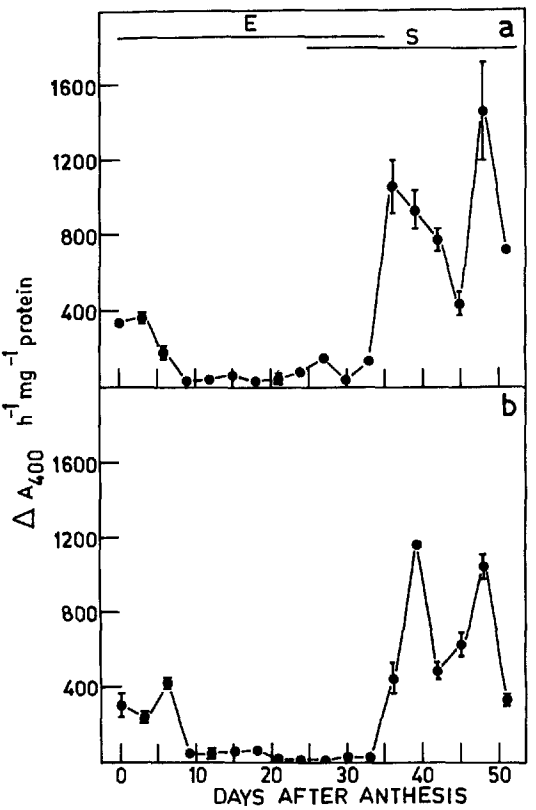

Fig. 2. Cytoplasmic (a) and salt-extracted (b) peroxidase activity with ferulic acid as hydrogen donor against boll age. Otherwise as for Fig. 1.

on the day of anthesis. Following an initial fall, a significant increase between d 6 and d 15 postanthesis was discernible, which clearly corresponded with the rate curve of elongation. After d 15 a gradual fall in esterase activity was observed, touching its lowest on d 27. From d 27 to d 39 of postanthesis the activity again showed an increasing trend. A negative relationship between the rate of dry matter accumulation and cytoplasmic esterase activity was discernible (Fig. 4a).

In the wall fraction also, nonspecific esterase activity maintained higher levels during the elongation phase and decreased during the secondary thickening phase (Fig. 4b). However, on d 39, when the boll opened, esterase activity again recorded a higher level.

Discussion

The epidermal cells of the cotton ovules elongate soon after anthesis, and for about half of their elongation phase they remain bounded by a thin primary wall. As the rate of elongation diminishes (after d 18 postanthesis), the rate of dry matter accumulation and secondary wall thickening increases. Thus elongation (d 3 to d 33) and secondary thickening (d 24 to d 51) phases appear as

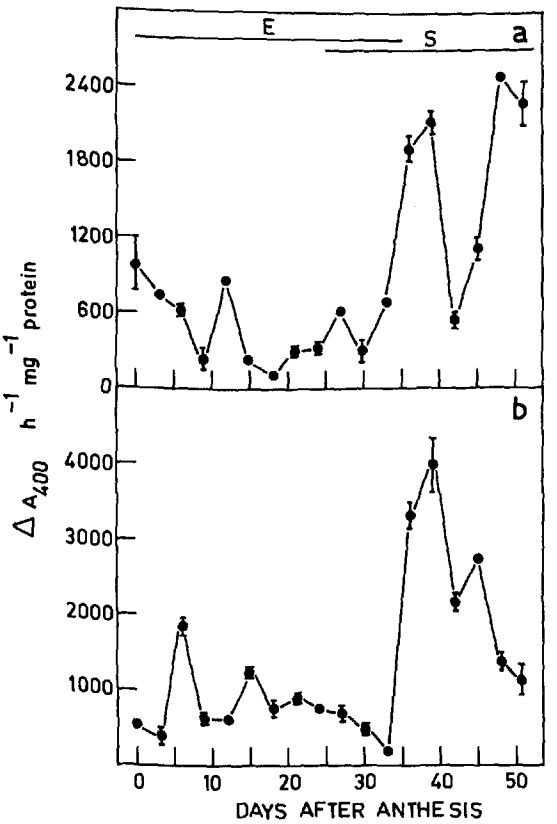

Fig. 3. Cytoplasmic (a) and salt-extracted (b) peroxidase activity with chlorogenic acid as hydrogen donor against boll age. Otherwise as for Fig. 1.

two distinct phases. However, a considerable overlap between these two phases was evident. Varying degrees of overlap between the elongation and secondary thickening phases have been reported (Jasdanwala et al. 1977, Naithani et al. 1982, Rama Rao et al. 1982a) in different cotton cultivars.

Cytoplasmic peroxidase activity with both ferulic acid (Fig. 2a) and chlorogenic acid (Fig. 3a) revealed that during the elongation phase the activity maintained low levels and that a sharp increase in the activity coincided with cessation of elongation growth. During the secondary thickening phase, peroxidase activity continued to maintain higher levels. Our earlier communications reported a comparable trend for IAA oxidase activity (Rama Rao et al. 1982a,b), and it was thus suggested that by regulating the endogenous level of IAA in cotton fiber, IAA oxidase system may play an important role in the cessation of elongation growth. However, in all these studies, cytoplasmic peroxidase activity assayed with guaiacol as the substrate showed considerable levels during the elongation phase, which is quite in contrast to the present study. Differences in the pattern of peroxidase activity with different phenol substrates have been reported in the developing pea seedlings (Gibson and Liu 1978). Further, a number of workers have demonstrated that peroxidase and IAA oxidase activities are associated with the same protein molecule (Siegel and Galston 1967, Srivastava and Van Huystee 1977, Stonier et al. 1979). A

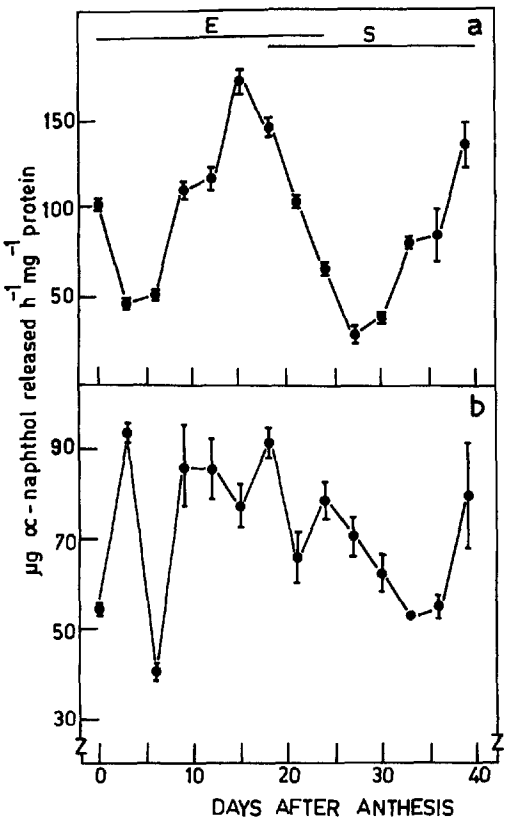

Fig. 4. Cytoplasmic (a) salt-extracted (b) esterase activity against boll age. Otherwise as for Fig. 1.

close parellelism between cytoplasmic peroxidase assayed with ferulic and caffeic acids as substrates, reported here, and IAA oxidase activity reported earlier (Rama Rao et al. 1982a,b) supported the view that the ability to oxidize IAA is one of the many capabilities intrinsic to peroxidase enzyme molecule.

Salt-extracted peroxidase activity also maintained low levels during the elongation phase, and it was only during the secondary thickening phase that the activity increased significantly (Figs. 2b, 3b), thus supporting the isodityrosine and diferulate cross-link models of cell expansion. Contrary to this, many tissues that do not undergo lignification or at least do not show the classical phloroglucinol lignin reaction, possess significant wall peroxidase activity (Hepler et al. 1972, Yung and Northcote 1975, Haddon and Northcote 1976), suggesting that this step may not be either a rate-limiting one or a site of control. Instead, Haddon and Northcote (1976) showed that phenylalanine ammonialyase, which produces precursors of lignin—i.e., phenolic alcohols, acts as a limiting step. However, it should be noted that cotton plant is known for its richness in phenolic compounds, and it is possible that the wall peroxidases in place of phenols may be acting as a rate-limiting step. Reviewing the work on peroxidases, Lamport (1980) suggested that peroxidase may slow down or limit cell expansion by (1) oxidizing free auxin (Schneider and Wightman 1974, Sembdner et al. 1980); (2) converting ferulic acid to diferulic acid, which

can act as a hemicellulosic cross-link (Markwalder and Neukom 1976, Whitmore 1976, Fry 1979); (3) generating hydrogen peroxide (via NADH-linked cell wall malate dehydrogenase) for the oxidation of cinnamoyl alcohols to their free redical lignin precursors (Gross et al. 1977, Mäder and Amberg-Fisher 1982); and (4) perhaps using phenolic amino acids to cross-link wall proteins (Fry 1982b).

Support for the phenol cross-link model is also apparent from our studies on nonspecific, wall-located esterases (Fig. 4b). These esterases maintained higher levels during the entire period of fiber elongation, whereas during the secondary thickening phase, their levels showed a decreasing trend, suggesting that these esterases may cleave the ester-linked phenols, which in turn may restrict the formation of cross-links. However, any speculation on the role of esterases in cell elongation must await further studies, and it should be clearly understood that nonspecific esterases consist of a large group of esterases, and unless one studies specific esterase(s) that hydrolyze the speculated phenolic esters, any conclusion would be pointless. Recently, Schöbel and Pollmann (1980) have isolated and characterized a specific esterase that cleaves the ester link between quinic acid and caffeic acid molecules of chlorogenic acid.

In the present work, nonspecific esterase activity in the cytoplasmic fraction (Fig. 4a) also recorded higher activity during the elongation phase whereas, during the secondary thickening phase, it decreased significantly. Sood (1974) also reported an increase in the nonspecific esterase activity during formation and elongation of the pollen tube. It has also been suggested that nonspecific esterases may play a vital role in the control of the overall energetics (Castillon et al. 1973) and primary wall synthesis (Gahan and McLean 1969).

Acknowledgments. We are thankful to Professor S. C. Pandeya for providing the necessary facilities and Dr. M. S. Murthy for going through the manuscript. This work was supported by the University Grants Commission, New Delhi.

References

Castillon MP, Catalan RE, Vega S, Municio AM (1973) Biochemistry of the insects *Dacus oleae* and *Ceratitis capitata*. Changes of cholinesterases and triglyceride hydrolyzing enzymes. Comp Biochem Physiol 44:639–646

Cooper JB, Varner JE (1983) Insolubilization of hydroxyproline rich cell wall glycoprotein in aerated carrot root slices. Biochem Biophys Res Commun 112:161–167

Fielding JL, Hall JL (1978) A biochemical and cytochemical study of peroxidase activity in roots of *Pisum sativum*. I. A comparison of DAB-peroxidase and guaiacol peroxidase with particular emphasis on the properties of cell wall activity. J Exp Bot 29:969–981

Fry SC (1979) Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. Planta 146:343–351

Fry SC (1982a) Phenolic components of the primary cell wall. Biochem J 203:493–504

Fry SC (1982b) Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. Biochem J 204:449–455

Gahan PB, McLean J (1969) Subcellular localization and possible function of acid β-glycerophosphatases and naphthol esterases in plant cells. Planta 89:126–135

Gardiner MG, Cleland R (1974) Peroxidase changes during the cessation of elongation in *Pisum sativum* stem. Phytochemistry 11:1095–1098

Gaspar T, Kevers C, Penel C, Greppin H (1983) Auxin control of calcium-mediated peroxidase secretion by auxin-dependent and auxin-independent sugarbeet cells. Phytochemistry 22:2657–2660

Gibson DM, Liu EH (1978) Substrate specificities of peroxidase isozymes in the developing pea seedling. Ann Bot 42:1075–1083

Gipson JR, Ray LL (1969) Fibre elongation rates in five varieties of cotton (*Gossypium hirsutum* L.) as influenced by night temperature. Crop Sci 9:339–341

Gross GG, Janes C, Elstner EF (1977) Involvement of malate, monophenols and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta 136:271–276

Haddon L, Northcote DH (1976) Correlation of the induction of various enzymes concerned with phenyl propanoid and lignin synthesis during differentiation of bean callus (*Phaseolus vulgaris* L.). Planta 128:255–262

Harris PJ, Hartley RD (1976) Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. Nature 259:508–510

Hartley RD, Jones EC (1976) Diferulic acid as a component of cell wall of *Lolium multiflorum*. Phytochemistry 16:1531–1534

Hepler PK, Rice RM, Terranova WA (1972) Cytochemical localization of peroxidase activity in wound vessel members of *Coleus*. Can J Bot 50:977–983

Jasdanwala RT, Singh YD, Chinoy JJ (1977) Auxin metabolism in developing cotton hairs. J Exp Bot 28:1111–1116

King EE (1971) Extraction of cotton leaf enzymes with borate. Phytochemistry 10:2337–2341

Lamport DTA (1980) Structure and function of plant glycoproteins. In: Preiss J (ed) The biochemistry of plants, Vol 3: Carbohydrates: Structure and function. Academic Press, New York, pp 501–541

Lamport DTA, Catt JW (1981) Glycoproteins and enzymes of the cell wall. In: Tanner W, Loewus FA (eds) Plant carbohydrates II: Extracellular carbohydrates. Springer-Verlag, New York, pp 133–167

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Mäder M, Amberg-Fisher V (1982) Role of peroxidase in lignification of tobacco cells. I. Oxidation of NAD and formation of H_2O_2 by cell wall peroxidases. Plant Physiol 70:1128–1131

Markwalder H-V, Neukom H (1976) Diferulic acid as a possible crosslink in hemicelluloses from wheat germ. Phytochemistry 15:836–837

Naithani SC, Rama Rao N, Krishnan PN, Singh YD (1981) Changes in O-diphenol oxidase during fiber development in cotton. Ann Bot 48:379–385

Naithani SC, Rama Rao N, Singh YD (1982) Physiological and biochemical changes associated with cotton fiber development. I. Growth kinetics and auxin content. Physiol Plant 54:225–229

Pantelouris EM (1968) A. Estimation of the esterase activity of serum. B. Starch-gel electrophoresis of mouse serum. In: Kerkut GA (ed) Experiments in physiology and biochemistry, Vol I. Academic Press, London, New York, pp 101–109

Rama Rao N, Naithani SC, Singh YD (1982a) Physiological and biochemical changes associated with cotton fibre development. II. Auxin oxidising system. Physiol Plant 55:204–208

Rama Rao N, Naithani SC, Jasdanwala RT, Singh YD (1982b) Changes in indoleacetic acid oxidase and peroxidase activities during cotton fiber development. Z Pflanzenphysiol 106:157–165

Ridge I, Osborne DJ (1970) Regulation of peroxidase activity by ethylene in *Pisum sativum*. Requirement for protein and RNA synthesis. J Exp Bot 21:720–734

Schneider EA, Wightman F (1974) Metabolism by auxin in higher plants. Annu Rev Plant Physiol 25:487–513

Schöbel B, Pollmann W (1980) Isolation and characterization of a chlorogenic acid esterase from *Aspergillus niger*. Z Naturforsch 35c:209–212

Sembdner G, Gross D, Liebisch H-W, Schneider G (1980) Biosynthesis and metabolism of plant hormones. In: MacMillan J (ed) Encyclopedia of plant physiology new series, Vol 9: Hormonal regulation of development. I. Molecular aspects of plant hormones. Springer-Verlag, Berlin, Heidelberg, New York, pp 281–444

Siegel SM, Galston AW (1967) Indole-acetic acid oxidase activity of apoperoxidase. Science 157:1557–1559

Smith MM, O'Brien TP (1979) Distribution of autofluorescence and esterase and peroxidase activities in the epidermis of wheat roots. Aust J Plant Physiol 6:201–219

Smith MM, Hartley RD (1983) Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr Res 118:65–80

Sood PP (1974) Histochemical studies on the distribution of non-specific esterase in the germinating pollen grains of *Portulaca grandiflora*. Biol Plant 16:102–106

Srivastava OP, Van Huystee RB (1977) IAA oxidase and polyphenol oxidase activities of peanut peroxidase isoenzymes. Phytochemistry 16:1527–1530

Stonier T, Stasions S, Murthy Reddy KBS (1979) The masking of peroxidase-catalysed oxidation of IAA in *Vigna*. Phytochemistry 18:25–28

Taiz L (1984) Plant cell expansion: Regulation of cell wall mechanical properties. Annu Rev Plant Physiol 35:585–657

Whitemore FW (1976) Binding of ferulic acid to cell walls by peroxidases of *Pinus elliottii*. Phytochemistry 15:375–378

Yung K-H, Northcote DH (1975) Some enzymes present in the walls of mesophyll cells of tobacco leaves. Biochem J 151:141–144