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A Critical Evaluation of Peroxidase Profiles in *Parthenium argentatum*

W. A. Cress,[1] R. Kadir,[1] and J. Van Staden[1]

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Specific localization of peroxidases after electrophoresis on nondenaturing polyacrylamide gels is discussed. The use of a multifunctional analysis for the separation of isoperoxidases from polyphenoloxidases is suggested.

KEY WORDS: peroxidase; polyphenoloxidase; guayule.

INTRODUCTION

The use of guayule (*Parthenium argentatum*) as an alternative source of rubber has been of interest for over 50 years (Taylor, 1947) and considerable effort has been expended in the area of guayule breeding. Breeding for improvement is more time-consuming than any other type of guayule research, as decades may be required to make substantial improvements in guayule rubber production using classic breeding techniques (Tysdal, 1980). Strategies for guayule improvement should include crossing and selection at the 36-chromosome (diploid) level to improve rubber accumulation. In addition, interspecific crosses between *P. argentatum* and *P. incanum, P. tomentosum, P. stramonium,* or *P. fruticosum* would be of benefit to increase disease resistance, cold resistance, and vigor (Tysdal, 1980).

The variation in reproductive strategies of guayule has decreased the effectiveness of breeding practices. Guayule can be diploid (36 chromosomes), which are self-incompatible, or polyploid (54, 72, or 108 chromo-

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[1] FRD/UN Research Unit for Plant Growth and Development, Department of Botany, University of Natal, P.O. Box 375, Pietermaritzburg 3200, Republic of South Africa.

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somes have been reported) and self-compatible but reproducing mainly by abnormal sexuality or apomixis. Guayule has the following reproductive strategies: amphimixis in which normal sexuality occurs (reduction followed by fertilization) or abnormal sexuality (nonreduction followed by fertilization); in addition, guayule can reproduce by apomixis with nonsegregating [nonreduced (2*n*)] pseudogamy, reduced (1*n*) pseudogamy, or segregating (pseudogamous) diplospory. These early cytological studies on guayule utilized cultivated progeny of seed collected in Mexico and Texas (Miller and Backhaus, 1986).

Because of the complexity of the reproductive methods utilized by guayule, it is necessary that a diploid (36-chromosome) line be selected for the study since factors such as penetrance may be involved in the phenotypic expression of peroxidase in polyploid guayule lines. For this reason the diploid guayule variety W10 was selected for all of the studies on the oxidase/polyphenoloxidase/peroxidase staining reactions.

Isozyme analysis of plant enzymes has become increasingly important in evolutionary studies of species divergence (Hurka *et al.*, 1989; Proctor *et al.*, 1989), in variation analysis of plant varieties (Brune and Van Lelyveld, 1984; Machado and Contel, 1989), and in plant breeding programs (Chaparro *et al.*, 1989). The rigorous use of isozymes in genetic analysis has led to important breakthroughs in the separation of diverging populations of a species (Brain, 1986). In recent years isoperoxidases have been used in such analyses (Brain, 1986, 1987). It was felt, therefore, that an isozyme analysis of guayule might provide an effective tool in the study of variation in guayule.

Due to the problems associated with the extraction of native plant proteins, there is some doubt as to whether the large number of isoperoxidases found in plants are native molecules. As some of these enzymes also exhibit oxidase/polyphenoloxidase activity, there is also the question whether they are isozymes of peroxidase, i.e., different enzymes with the same function, and should therefore be regarded as peroxidases.

Additionally recent work (Stevens and Van Huystee, 1981; Hu and Van Huystee, 1989) raises the question whether there are multiple forms of some peroxidases or whether there are only a few isozymes and the rest arise from posttranslational causes or are extraction artifacts. Unless it can be established whether these enzymes arise from multiple alleles at a single genetic locus (isozyme) or multiple genetic loci (allozyme), their proper designation becomes problematic. One method of unequivocally establishing the presence of isozymes (allozymes) is a comparison of peptide maps of tryptic digests. This requires that the peroxidases be purified to homogeneity but does not provide any information on enzyme specificity. While these problems with the analysis of peroxidase isozymes are well known, a

standardized method for the rapid study of peroxidase variation within a single species is highly desirable. This is of particular importance in genetic analyses of relatedness, especially if isozymes of peroxidase are being identified based upon a substrate-linked staining reaction in electrophoretic gels.

While the purification and characterization of each individual isozyme or allozyme remain a long-term goal, there remains a need for more immediate methods to recognize relatedness. During the course of our investigations on peroxidases in *Parthenium argentatum,* it became evident that the recommendations of Van Loon (1971) were not adequate for the separation of peroxidases from polyphenoloxidases in our system. This paper examines some of the problems associated with peroxidase isozymes in genetic studies on relatedness.

Since isozyme studies are meaningful only if the substrate-linked staining reactions are specific for a single enzyme and its isozymes, we have demonstrated the inconsistencies in the methods currently used in studies on peroxidase isozymes.

MATERIALS AND METHODS

Plant Material

Composite samples of mature leaf tissue were collected from each of five 2-year-old guayule *Parthenium argentatum* Gray (W10, a diploid variety) plants. Alternatively mature leaf tissue was collected from 4-month-old seedlings of varieties W5, W6, W10, W13, and W16 and the hybrid A101 (*Parthenium argentatum* × *P. tomentosum* var. *stramonium*) grown and maintained according to the techniques of Madhavan and Benedict (1984).

Tissue Preparation

Fresh material (0.5 g) from mature leaf tissue from each individual plant was homogenized in a chilled mortar with 2 ml of extraction buffer (Brain, 1986) containing 0.5 g of polyvinylpyrrolidone. The extraction buffer was modified by the elimination of 2-mercaptoethanol, sodium dodecyl sulfate, and bromophenol blue. The homogenate was centrifuged for 20 min at a setting of 10 in a Hettich EBA III centrifuge, and the supernatant collected and stored at 4°C. The enzymatic activity of extracts toward benzidine was stable for up to 2 months when stored at 4°C.

Electrophoresis

Extracts were electrophoresed on 12% nondenaturing discontinuous acrylamide gels with a 1-cm stacking gel (Davies, 1964). Electrophoresis was carried out at a constant 75 V during stacking and at 134 V for 5 hr during separation.

Enzyme Stains

Gels were stained for enzymatic activity immediately after the completion of each electrophoretic run. Tyrosinase activity was visualized with catechol/L-proline (Shaw and Parsid, 1970), polyphenoloxidase (or laccase) with caffeic acid/*m*-phenylenediamine, with and without the addition of catalase (Van Loon, 1971), catechol/sulfanilic acid (Vallejos, 1983), and oxidase with α-naphthol in 10% ethanol (Haas and Hill, 1928). Peroxidase activity was visualized using benzidine/H_2O_2 (Brain, 1986), pyrogallol/H_2O_2 (Haas and Hill, 1928), and guaiacol/H_2O_2 (Staples and Stahmann, 1964). The following modification of the benzidine peroxidase (Brain, 1986) assay was used. Ascorbic acid (0.1%), an inhibitor of polyphenoloxidase activity, was added to the benzidine solution. In addition, the electrophoresed gel was preincubated in 0.1% ascorbate prior to staining for peroxidase activity. The R_f's were determined for three replicates for each staining reaction on freshly stained gels.

RESULTS

When protein extracts of mature leaves from five adult plants were run on nondenaturing gels and treated with the assay systems or stains currently in use for peroxidases, considerable variation between methods was observed. Guaiacol is the classic spectrophotometric peroxidase assay, while benzidine has come into common use in assays and in polyacrylamide gel stains.

The following results were obtained: guaiacol produced five bands with R_f's of 0.18, 0.30, 0.36, 0.37, and 0.38; benzidine produced five or six bands with R_f's of 0.18, 0.29, 0.30, 0.33, 0.36, and 0.38; and benzidine/ascorbate produced the same banding pattern as guaiacol with respect to both the number of bands and the R_f's. Staining with pyrogallol produced six or eight bands having the R_f's 0.02, 0.03, 0.05, 0.18, 0.31, 0.34, 0.36, and 0.39.

Additionally, stains for polyphenoloxidase, laccase, and tyrosinase activity, all of which are polyphenoloxidases, and the oxidase stain were compared with those obtained using the stains for peroxidase activity. A comparison of all the "peroxidase" R_f's (Fig. 1) among the different peroxidase stains and between the various polyphenoloxidase stains reveals the inconsis-

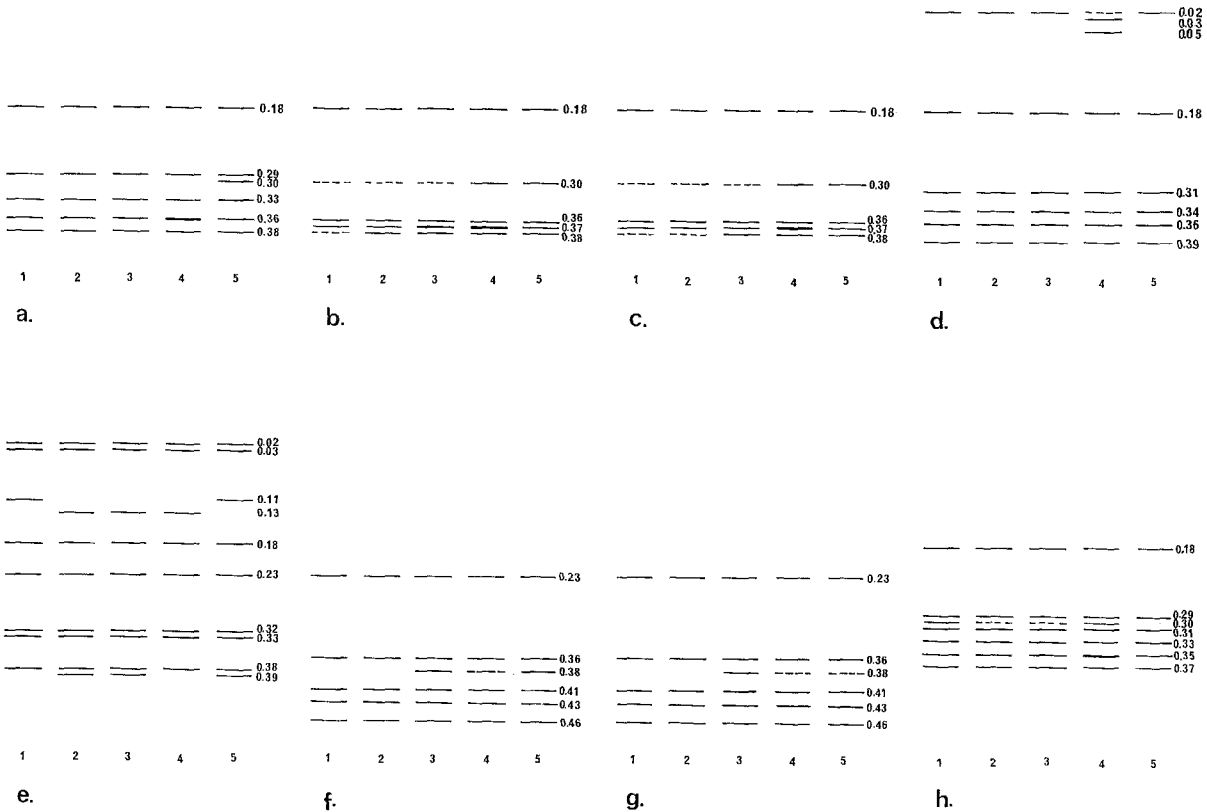

Fig. 1. A comparison of R_f's of the various staining reactions of the peroxidase, oxidase, and polyphenoloxidase staining reactions of protein extracts of mature leaves from 2-year-old guayule (variety W10). Each lane (1–5) represents the same plant in each staining reaction: (a) benzidine; (b) benzidine + ascorbic acid; (c) guaiacol; (d) pyrogallol; (e) caffeic acid + *m*-phenylenediamine or caffeic acid + *m*-phenylenediamine + catalase; (f) catechol + sulfanilic acid; (g) catechol + L-proline; (h) α-naphthol + H_2O_2.

tencies of the peroxidase staining procedures currently in use. The R_f 0.18 and 0.38 bands were observed with all of the staining procedures. The use of ascorbate and catalase (Van Loon, 1971) did not resolve whether they were peroxidases or polyphenoloxidases.

Both similarities and differences were observed using the polyphenoloxidase stain: caffeic acid/*m*-phenylenediamine, in both the presence and the absence of catalase, produced eight or nine bands having R_f's of 0.02, 0.03, 0.11, 0.13, 0.18, 0.23, 0.32, 0.33, 0.38, and 0.39; the laccase stain catechol/sulfanilic acid produced five or six bands with R_f's of 0.23, 0.36, 0.38, 0.41, 0.43, and 0.46. The tyrosinase stain catechol/L-proline gave the same R_f's as the laccase stain. The oxidase stain α-naphthol/H_2O_2 produced six or seven bands with the R_f's 0.18, 0.29, 0.30, 0.31, 0.33, 0.35, and 0.37.

Figure 2 shows the difference in both number of bands and R_f's observed for mature leaves of five individual 4-month-old guayule seedlings. No single R_f peroxidase band was observed in common in the varieties examined. An R_f 0.33 peroxidase band and an R_f 0.35 band were observed in

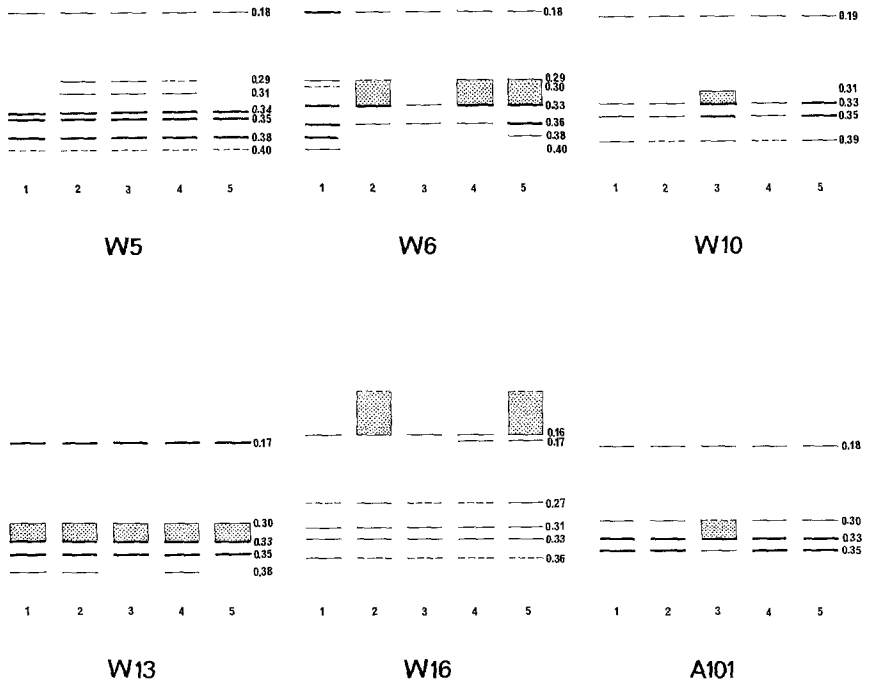

Fig. 2. A comparison of the benzidine plus ascorbate plus hydrogen peroxide staining reaction R_f's of guayule protein extracts electrophoresed in nondenaturing acrylamide gels. Each lane (1–5) represents a single individual of the guayule varieties W5, W6, W10, W13, W16, and the hybrid A101.

four of the six varieties. If we compare the peroxidase banding patterns of the W10 variety of the 4-month-old plants with that of the 2-year-old W10 variety, we see that an age effect is also present.

DISCUSSION

A comparison of the peroxidase staining reactions illustrates the inadequacies of the various "peroxidase" staining procedures currently in use. For this reason it is important in peroxidase isozyme work to standardize the procedures used in distinguishing between peroxidases and polyphenoloxidases. In the current work we have demonstrated different staining (i.e., substrate) specificities depending on the substrate used (Fig. 1). This is illustrated by the appearance of bands at new R_f's when the substrate is changed in the various staining reactions. Of further importance as regards specificity is whether H_2O_2 is required as a substrate in the reaction or whether molecular O_2 will act as a substrate. Of particular importance in our interpretation is the distinction between what constitutes a peroxidase reaction and what constitutes an oxidase/polyphenoloxidase reaction. The peroxidase reaction is an oxidoreductase acting on H_2O_2 as the terminal electron acceptor (i.e., donor + H_2O_2 = oxidized donor + $2H_2O$). Oxidase/polyphenoloxidase reactions are oxidoreductases acting on diphenols and related substrates as electron donors with molecular oxygen as the terminal electron acceptor in the reaction (i.e., 4 benzenediol + O_2 = 4 benzosemiquinone + $2H_2O$).

While isozymes may have different substrate affinities, they should not have different substrate specificities. In other words, they should not recognize entirely different substrates or unrelated chemical structures. The results illustrated in Fig. 1 suggest that all of the "isozymes" observed are not isozymes of peroxidase but are enzymes of different specificities and therefore react with a variety of substrates having structural similarities or can function in redox reactions and thus would not qualify as isozymes of peroxidase. Badiani *et al.* (1990) have demonstrated in winter wheat the presence of isoperoxidases which have phenoloxidase activities. In the present case all of the isozymes demonstrate both peroxidase and phenoloxidase activity (Badiani *et al.,* 1990). These results therefore suggest that a multifunctional analysis is even more important if isozymes of peroxidases are to be separated from polyphenoloxidases, particularly if some peroxidases are multifunctional in nature.

With respect to substrate specificities similarities between the peroxidase staining reactions and the oxidase/polyphenoloxidase reactions were observed in the present study. When comparisons between the various staining reactions were made, the R_f 0.18 band indicated a lack of substrate

specificity for this enzyme, both within the peroxidase reactions and between the peroxidase and the polyphenoloxidase/oxidase reactions (Fig. 1). This enzyme is able to react with all of the peroxidase substrates and two of the oxidase/polyphenoloxidase substrates. This lack of substrate specificity between peroxidase/oxidase/polyphenoloxidase staining reactions would indicate an enzyme of low substrate specificity, which is thus unlikely to be a simple isozyme of peroxidase. It may, however, be an enzyme with both peroxidase and polyphenoloxidase activity. If it were a simple isozyme, why do we not see a cross-reactivity reflected among all the substrates used in the staining reactions? We therefore suggest that if "peroxidase" isozymes are to be used in studies on genetic variation, and if more vigorous methods of isozyme identification cannot be performed, then they should be used as phenotypes rather than genotypes and their isozyme status should be characterized with respect to substrate specificity in a peroxidase/oxidase/polyphenoloxidase multifunctional system.

Thus a careful analysis with the peroxidase/oxidase/polyphenoloxidase staining system as described here should reveal those enzymes which are not peroxidases or are multifunctional in nature. If we, further, add ascorbic acid—a powerful inhibitor of polyphenoloxidase—to our peroxidase reaction (Fig. 1b) and catalase to our polyphenoloxidase reactions (Van Loon, 1971), we should be able to distinguish between the peroxidase and the oxidase/polyphenoloxidase reactions. In other words, to make this distinction with respect to what is a peroxidase, we should compare peroxidase staining reactions with the oxidase/polyphenoloxidase staining reactions in the presence of ascorbic acid.

Results using benzidine plus ascorbate as a stain on mature leaves of 4-month-old seedlings (Fig. 2) showed considerable variation both within and between the varieties of *P. argentatum* and the single hybrid of *P. argentatum* × *P. tomentosum* var. *stramonium*) and, also, indicated that no single R_f was represented in all of the varieties. An R_f 0.33 peroxidase band and an R_f 0.35 band were observed in four of the six varieties (Fig. 2). To confuse our interpretation further, both of these R_f's occurred in the mature W10 variety as oxidase/polyphenoloxidase-reacting enzymes, but except for the benzidine plus H_2O_2 staining reaction these R_f's were not present in the "peroxidase" reactions. Thus we must conclude that they are not isozymes of peroxidase, although they may be multifunctional enzymes with both peroxidase and oxidase activity (Badiani *et al.*, 1990).

The increase in number of peroxidase "isozymes" in mature versus immature tissue is a well-known phenomenon. This variation in number of isozymes between mature and immature tissue should be carefully analyzed with the oxidase/polyphenoloxidase/peroxidase system to determine which are of low specificity and do not meet the criteria of a peroxidase and

therefore are unlikely to be isozymes of peroxidase. We therefore recommend that for isozyme studies of seedling peroxidases, a system of peroxidase/oxidase/polyphenoloxidase stains should be used in conjunction with the recommendations of Van Loon (1971) to determine the specificity of the isozymes of peroxidase, and the criterion of H_2O_2 as the terminal electron acceptor should be utilized in the recognition of peroxidase isozymes. Additionally if these methods are followed, then multifunctional peroxidases (Badiani *et al.,* 1990) can be separated from both the single functional peroxidase and the oxidase/polyphenoloxidase isozymes.

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