Phytochemistry, Vol. 21, No. 8, pp. 1843–1847, 1982.
Printed in Great Britain.

0031-9422/82/081843-05$06.00/0

REVIEW

PROGRESS AND PROSPECTS IN THE USE OF PEROXIDASE TO STUDY CELL DEVELOPMENT

R. B. VAN HUYSTEE and W. L. CAIRNS*

Departments of Plant Sciences; and *Chemical and Biochemical Engineering, University of Western Ontario, London, Ontario, Canada N6A 5B7

(*Revised received* 26 *January* 1982)

Key Word Index—Peroxidase; synthesis; isozymes; interspecies relationship; cell development.

Abstract—The need for peroxidase purification is stressed as a requirement for comparative studies on isoenzyme structure as well as for detailed investigations on biosynthesis. A single cationic protein possessing the major peroxidase activity was isolated from the medium in which peanut cells had grown. The antibodies raised against this pure protein were employed as a probe to study the site of synthesis of peroxidase in the cell as well as the proportion of total synthesized protein which was peroxidase. Structural studies on the purified isoenzymes suggest the presence of three gene loci for peroxidase in cultured peanut cells. The results are discussed together with potential assays for induction of this enzyme and the relationship to cell development.

It is generally accepted that peroxidase activity and/or its isoenzyme patterns alter with changes in plant development[1–3]. Various functions in cell development have been assigned to peroxidase[3–6] but its involvement in lignin synthesis has been most commonly stressed[7–9]. In spite of the reported relationship between cell development, and the activity and isoenzyme patterns of peroxidase, there has yet to be published a detailed study on the induction of this enzyme. The work carried out on enzymes involved in phenolic biosynthesis[10] illustrates the feasibility of obtaining a detailed understanding of complex regulatory mechanisms in plant systems.

Perhaps the major handicap for peroxidase studies has been the lack of controls[11]. In several instances, artifacts obtained through staining[7] or cytoplasmic disruption[12] have been suggested as specific problems, which could be interpreted as post-translational alterations and/or difficulties in identification of the true peroxidase [3]. Without being able to distinguish the true and artificial peroxidases, detailed studies on biosynthesis are probably futile.

In most modern studies, antibodies raised against a pure product are used in *in vitro* studies to follow synthesis of the specific protein under investigation [10, 13–15]. Yet only a few reported cases of peroxidase isolation exist[16–19]. In the case of cytochrome *c* peroxidase, purification has proceeded to crystalline form[20]. While this purity may be desired for a detailed study by crystallography[20], a comparison of isoenzyme forms can be made from amino acid analysis following hydrolysis of electrophoretically homogeneous proteins having peroxidase activity[21]. Alternatively, comparisons of isoenzyme structure may be made by immunological assays [22, 23] or by peptide mapping [16, 24]. Whatever the approach used, purity is of major importance.

Isolation of a pure protein which possesses an exceptionally large MW or which is present in abundance in the cell, is relatively simple[25–27]. Conversely, proteins such as peroxidase, which are prone to post-translational alterations during purification steps and which are normally present in small amounts are more difficult to isolate in pure primary forms[3]. The following is a description of a rapid purification of peroxidase and subsequent studies on its biosynthesis.

It was observed early in studies with cultured peanut cells grown in suspension medium that five so-called anionic isoenzymes of peroxidase could be detected by peroxidase specific staining of gels following electrophoresis. These five occurred in the cell extract as well as in the medium that had supported growth of these cells [28, 29]. The five isoenzymes could be reduced to one form in the cell extract by extraction in the presence of 0.1% bovine serum albumin [30] and in the medium by treatment with Dowex resin [29]. The conclusion drawn from this and the literature was that post-synthetic alterations may be brought about through phenolic interactions [30] Recently this single anionic protein with peroxidase activity has been purified from the medium as shown below.

Peroxidase appears to be selectively and rapidly released into the medium by cultured peanut cells[31]. The selectivity of release is also demonstrated by the high specific enzyme activity found in the medium as compared to that in the crude cell extract [32]. Labelled protein patterns from cells incubated for 2 hr with [^{35}S]methionine were compared with protein patterns from the medium following 6

days of incubating the cells. The fluorographs prepared after electrophoresis of cell extract and proteins from the medium showed numerous labelled proteins in the former but only a few in the latter [33]. Protease activity could not be detected in the medium [31, 34], and therefore protein degradation could not be considered as the cause for the few proteins in the medium. It has been suggested that plant cells restrict the release of cellular components [35]. The reason for the preferential release of peroxidase may be sought in its extracellular function as a mediator in lignin synthesis [7, 9, 36]. The occurrence of lignin in cultured plant cells and in their medium has been noted [34, 37, 38]. For isolation purposes the selective release of peroxidase by the cells, makes the medium an ideal source. By relatively few manipulations a single cationic protein with nearly 3/4 of all peroxidase activity in the medium could be isolated [39]. The purity of the single protein was demonstrated by electrophoresis on polyacrylamide gels containing SDS, by the high specific enzyme activity, by the high RZ (A_{403}/A_{280} nm) value of this hemoprotein [39] as well as by immunoelectrophoresis [23]. Antibodies were raised against this purified cationic peroxidase (MW 44 000) and the antibodies were used as a specific probe for peroxidase biosynthesis.

The necessity for a specific probe was demonstrated in initial studies on *in vivo* peroxidase biosynthesis with radioactive amino acids. Labelled proteins were found in the medium [29, 31, 33] but specific peroxidase identification could not be made, due to contaminating proteins which prevented electrophoretic homogeneity. This problem of identification is aggravated in a cell extract, or by *in vitro* synthesis with isolated polysomes using the S-30 wheat germ fraction [33].

The technique of immunoprecipitation is based on the very specific interaction of the antigen with its own antibody. This technique has been used with success on proteins more abundant than peroxidase in the cell [13, 15, 40]. This specific removal of one protein from amongst all other *in vitro* synthesized proteins was also applied to peroxidase with the antibodies raised against the major cationic peroxidase. The electrophoresis of the resuspended immunoprecipitate on a gel showed by fluorography a single radioactive band with a R_m approximately the same as that of the originally purified peroxidase used to raise the antibodies [33]. These results suggested that additional studies employing immunoprecipitation were feasible. These studies were primarily directed towards finding the site of synthesis of the apoprotein moiety for peroxidase, and the proportion of all newly synthesized proteins that is peroxidase.

Studies on the membrane fractions of cultured peanut cells had shown that relatively high concentrations of peroxidase were associated with the rough endoplasmic fraction [41]. The presence of peroxidase in this fraction had been ascribed to a possible secretory process for peroxidase. In animal cells, secretory proteins are synthesized on the rough endoplasmic reticulum [42]. The immunoprecipitation with antibodies against peroxidase could now be applied to the resolution of this problem in peanut cells. Free and membrane polysomes were separated by differential centrifugation and purified [33]. Each polysome population was used for *in vitro* protein synthesis assays followed by trichloroacetic acid precipitation and immunoprecipitation to measure total protein synthesis and peroxidase synthesis respectively. The data indicate that the majority of peroxidase was synthesized on the membrane polysomes [43]. Consistent with this observation is the glycoprotein nature of peroxidase [16, 23, 44]. Glycoprotein synthesis is aided by dolichol oligosaccharides [45] and the glycosylation enzyme dolichol phosphokinase has been shown to be associated with rat microsomes [46]. These observations, together with the report that *in vitro* synthesis and coupled glycosylation of the glycoprotein, lactalbumin, has been obtained in the presence of the microsomal fraction [14] suggest that peroxidase may follow the same route of synthesis. Moreover secreted proteins in animals have been shown to employ the Golgi apparatus for ferrying them across the cytoplasm [42]. This transport can be arrested by respiratory inhibitors such as antimycin A [47]. Recent results (unpublished) on the transport of peroxidase monitored by immunoprecipitation have shown that 10^{-5} M antimycin A also abolishes the release of peroxidase by cultured peanut cells. Therefore, it is probable that peroxidase release in peanut cells commences at the rough endoplasmic reticulum and proceeds via the Golgi apparatus as for glycoproteins in animal cells [48].

The question of what fraction of the total protein synthesized by the cell is peroxidase still remains to be answered. In preliminary steps, the poly (A) RNA, representing *m*RNA, was separated by oligo (dT) cellulose chromatography from total RNA [43]. The poly (A) RNA was used as templates for *in vitro* protein synthesis. Comparisons of the radioactivity from the immunoprecipitate to that of the trichloroacetic acid precipitate suggested that 2% of overall protein synthesis is destined for peroxidase biosynthesis [43]. This value appears to be reasonable in light of the requirement not only for intracellular but also extracellular peroxidase in the medium. Two per cent is not excessive in comparison to synthesis of another plant enzyme, cellulase [49].

It should be recalled that all the immunoprecipitation was carried out with antibodies raised against the major cationic peroxidase isozyme. In the purification of the protein used to raise antibodies it was noted that some peroxidase was washed through the column in the 'flow through' fraction and some peroxidase was left on the CM cellulose column after elution of the major cationic peroxidase [39]. Are these two minor fractions different isoenzymes of the major peanut peroxidase or are they post-translational alterations of the major form? Each minor fraction was purified further; the anionic form by DEAE cellulose and the cationic form by rechromatography on CM cellulose at a higher salt gradient. Two pure protein fractions were obtained, each with peroxidase activity. The two cationic isozymes have identical MWs of 44 000. The anionic form has a MW of 46 000 [43]. Protease alteration of the MWs was discounted, due to the absence of protease in the medium [31, 34] and the inability of performic acid treatment to affect the MW [23]. Fur-

thermore, no immunological relatedness could be established for the three peroxidases [23]. The technique of peptide mapping has been used in comparisons of structural relationships for other plant proteins [13, 15]. Therefore the three peroxidase isoenzymes in the presence of SDS were incubated with protease from *Staphylococcus aureus* and the resulting peptide sequences analysed by SDS-PAGE. The patterns of the peptides obtained for each isoenzyme were totally different from each other [43]. These differences warrant the assumption that three gene loci occur in cultured peanut cells for peroxidase. This is in good agreement with the data on peroxidase from horseradish, wheat and tomato even though these data were largely obtained by different procedures [50–52]. The role of these isoenzymes in the plant cell has not yet been established but they may play a role in lignin synthesis, as suggested for tobacco [53].

Having considered differences between isoenzymes of peroxidase in one plant species, the question of interspecies differences should be considered. The major peanut cationic peroxidase against which antibodies has been raised did not exhibit any immunological relatedness to that from horseradish by either immunoelectrophoresis [23] or by immunoaffinity chromatography [54]. These observations were not surprising since interspecies differences have also been observed with turnip [55] and tobacco [56] peroxidases.

It is possible to look ahead and suggest some studies which truly establish the relationship with and perhaps function of peroxidase in cell development. In concert with other scientists who have been interested in induction of enzymes, we have suggested earlier that either phytochrome and/or heme concentrations may govern the induction of peroxidase synthesis [3]. Phytochrome has a definite control over the synthesis of the apoprotein moiety of the light harvesting complex [57]. Since the light harvesting complex is a porphyrin–protein molecule, and so is peroxidase, it may be possible that phytochrome has control over peroxidase synthesis [3]. However, a study on two enzymes of phenolic metabolism derived from cultured plant cells have shown that they are induced either by UV irradiation [10] or cell density dilutions [58]. Therefore enzymes may be induced by agents other than phytochrome. But in light of the hemoprotein nature of peroxidase it remains equally possible that heme concentrations regulate the synthesis of peroxidase, as suggested earlier [3]. Heme controls the major enzymes of porphyrin synthesis [59, 60]. Amino triazole through its effect on heme concentrations affects protein synthesis on mitochondrial polysomes [61]. It appears also pertinent to refer to the synthesis of another hemoprotein, cytochrome P450, which is most effective in the presence of a mitochondria–rough endoplasmic reticulum complex [62]. These observations suggest that the activity of the mitochondria and particularly its side product, the heme, may have an influence on peroxidase induction.

Following studies to identify inducing agents, clarification of the role of peroxidase in cell development requires the linking of the period of induction to a specific stage of cell development. Cell development is often tied to hormonal control and therefore related to changes brought about by enzymes induced by hormones. As an example of this approach, the developmental state brought about by gibberellic acid may be considered in the light that induction of poly (A) RNA for amylase occurs 12 hr after application of gibberellic acid [63]. The onset for the *m*RNA of amylase was assayed by *in vitro* synthesis and separation of the proteins produced by one-dimensional electrophoresis. A more precise identification of proteins, particularly when not present in high concentrations is by two-dimensional (2D) electrophoresis. This technique has been used to indicate the synthesis and deletion of as yet unidentified peptides in response to auxins [64].

Various forms of nucleic acid hybridization are rapidly becoming the most elegant techniques used to detect and quantitate the induction of *m*RNA. On a large but precise scale this technique has permitted the determination of the percentage of single-copy DNA which participates in the formation of a mature tobacco plant [65]. On a small scale it has been possible to predict the number of new peptides that would be involved in the formation of a nodule from an ordinary legume root [66]. Later it was possible by immunoprecipitation and 2D electrophoresis to identify the 20 new peptides [67]. Nevertheless the most promising use of hybridization is the use of recombinant cDNA as a probe to search for the presence of *m*RNA. The cDNA is derived by incubation of purified *m*RNA with reverse transcriptase. The purified *m*RNA is obtained by density centrifugation of total poly (A) RNA and perhaps further electrophoresis to obtain an enriched *m*RNA fraction. Each step is monitored for the specific *m*RNA by *in vitro* synthesis and immunoprecipitation. Using the cDNA for tubulin it was possible to determine the induction of the *m*RNA in *Chlamydomonas* in terms of min instead of hr [68]. If this procedure could be used for peroxidase in a cell culture with an enhanced synthesis as shown above, the results would be incomparably more accurate than using inhibitors of RNA transcription [41].

In conclusion a few cautionary notes should be raised. Particularly when employing cell cultures, some genetic alterations may have arisen since making the explant. It has been shown that some peroxidase in the peanut leaf is immunologically related to that in cultured cells [22]. Conversely cultured plant cells do not contain chlorophyll [3]. While the cause for this loss is not yet known it has been shown that the cells no longer produce *m*RNA for the apoprotein moiety of light harvesting complex [69]. Loss of accumulation of *m*RNA may occur also in whole plants as shown with the loss of the *m*RNA for the ribulose bi-phosphate carboxylase in the mesophyll cells of corn [70]. Even if enough *m*RNA is present, the synthesis of the protein may not occur. The best known example here is the control of heme over the translation of the *m*RNA for globin in erythrocyte cells [71, 72]. The question of whether heme controls the induction of peroxidase synthesis was raised above and earlier [3].

With the above progress having been made towards a more detailed study of peroxidase, it may be possible to proceed with a more precise analysis of the

association between peroxidase and cell development. It would be of considerable benefit to all interested in cell development if one of the many subtle changes leading to cell differentiation could be described in greater detail. This analysis would be timely with a fresh evaluation of the role of plant growth regulators also under way [73]. In addition, studies on purified peroxidase may serve as a model for the nature and biosynthesis of other commonly found hemoproteins or glycoproteins.

REFERENCES

1. Schneider, E. A. and Wightman, F. (1974) *Annu. Rev. Plant Physiol.* **25**, 503.
2. Catesson, A.-M. (1980) *Ber. Dtsch. Bot. Ges.* **93**, 141.
3. van Huystee, R. B. and Cairns, W. L. (1980) *Bot. Rev.* **46**, 429.
4. Hösel, W., Frey, G. and Barz, W. (1975) *Phytochemistry* **14**, 417.
5. Grob, K. and Matile, Ph. (1980) *Z. Pflanzenphysiol.* **98**, 235.
6. Whitmore, F. W. (1978) *Plant Sci. Letters* **13**, 241.
7. Stafford, H. A. (1974) *Recent Advances in Phytochemistry* Vol. 8, p. 72. Plenum, New York.
8. Hahlbrock, K. and Grisebach, H. (1979) *Annu. Rev. Plant Physiol.* **30**, 109.
9. Fry, S. C. (1979) *Planta* **146**, 343.
10. Schröder, J., Kreuzaler, F., Schäfer, E. and Hahlbrock, K. (1979) *J. Biol. Chem.* **254**, 57.
11. Scandalios, J. G. (1974) *Annu. Rev. Plant Physiol.* **25**, 251.
12. Gibson, D. M. and Liu, E. H. (1981) *J. Exp. Botany* **32**, 419.
13. Apel, K. and Kloppstech, K. (1978) *Eur J. Biochem.* **85**, 581.
14. Lingappa, V., Lingappa, J. R., Prasad, R., Ebener, K. E. and Blobel, G. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2338.
15. Hall, T. C., Ma, Y., Buchbinder, B. U., Pyne, J. W., Sun, S. M. and Bliss, F. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3196.
16. Welinder, K. G. (1979) *Eur. J. Biochem.* **96**, 483.
17. Takio, K. and Yonetani, T. (1980) *Arch. Biochem. Biophys.* **203**, 605.
18. Mazza, G. and Welinder, K. G. (1980a) *Eur. J. Biochem.* **108**, 473.
19. Morita, Y. and Ida, S. (1968) *Agric. Biol. Chem.* **32**, 441.
20. Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Erikson, B., Xyong, N., Yonetani, T. and Kraut, J. (1980) *J. Biol. Chem.* **225**, 575.
21. Shannon, L. M., Kay, E. and Lew, J. Y. (1966) *J. Biol. Chem.* **241**, 2166.
22. Cairns, E., van Huystee, R. B. and Cairns, W. L. (1980) *Physiol. Plant.* **49**, 79.
23. van Huystee, R. B. and Maldonado, B. A. (1982) *Physiol. Plant.* **54**, 88.
24. Kim, S. S., Wender, S. H. and Smith, E. C. (1980) *Phytochemistry* **19**, 169.
25. Goldthwaite, J. J. and Bogorad, L. (1971) *Analyt. Biochem.* **41**, 57.
26. Gray, J. C. and Wildman, S. G. (1976) *Plant Sci. Letters* **6**, 91.
27. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15**, 3.
28. Verma, D. P. S. and van Huystee R. B. (1970) *Can. J. Biochem.* **48**, 444.
29. Srivastava, O. P. and van Huystee, R. B. (1977) *Bot. Gaz.* **138**, 457.
30. van Huystee, R. B. (1977) *Z. Pflanzenphysiol.* **84**, 427.
31. van Huystee, R. B. and Turcon, G. (1973) *Can . J. Botany* **51**, 1169.
32. Kossatz, V. C. and van Huystee, R. B. (1976) *Can. J. Botany* **54**, 2089.
33. Stephan, D. and van Huystee, R. B. (1980) *Can. J. Biochem.* **58**, 715.
34. van Huystee, R. B. (1976) *Bot. Gaz.* **137**, 325.
35. Zenk, M. (1978) in *Frontiers of Plant Tissue Culture* (Thorpe T. A., ed.), p. 6. University of Calgary Printing, Calgary.
36. Alibert, G., Ranjeva, R. and Boudet, A. M. (1977) *Physiol. Vég.* **15**, 279.
37. Moore, Th. S., Jr. (1973) *Plant Physiol.* **51**, 529.
38. Nimz, H., Ebel, J. and Grisebach, H. (1975) *Z. Naturforsch.* **30**, 442.
39. Maldonado, B. A. and van Huystee, R. B. (1980) *Can. J. Botany* **58**, 2280.
40. Bollini, R. and Chrispeels, M. J. (1979) *Planta* **146**, 487.
41. van Huystee, R. B. (1978) *Phytochemistry* **17**, 191.
42. Palade, G. (1975) *Science* **189**, 347.
43. Stephan, D. and van Huystee, R. B. (1981) *Z. Pflanzenphysiol.* **101**, 313.
44. van Huystee, R. B. (1976b) *Can. J. Botany* **54**, 876.
45. Hsu, A. F., Baynes, J. W. and Heath, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2391.
46. Rip, J. W. and Carroll, K. K. (1980) *Can. J. Biochem.* **58**, 1051.
47. Jamieson, J. D. and Palade, G. E. (1968) *J. Cell Biol.* **39**, 589.
48. Brodie, D. A. (1981) *J. Cell Biol.* **90**, 92.
49. Verma, D. P. S., Maclachlan, G. A., Byrne, H. and Ewings, D. (1975) *J. Biol. Chem.* **250**, 1019.
50. Shih, J. H. C., Shannon, L. M., Kay, E. and Lew, J. Y. (1971) *J. Biol. Chem.* **246**, 4546.
51. Kobrehel, K. (1978) *Can. J. Botany* **56**, 1091.
52. Rick, C. M., Zobel, R. W. and Fobes, J. F. (1974) *Proc. Natl. Acad. Sci., U.S.A.* **71**, 835.
53. Mäder, M., Ungemach, J. and Schloss, P. (1980) *Planta* **147**, 467.
54. Lobarzewski, J. and van Huystee, R. B. (1982) *Plant Sci. Letters* (in press).
55. Mazza, G. and Welinder, K. G. (1980b) *Eur. J. Biochem.* **108**, 481.
56. Mäder, M. (1980) *Z. Pflanzenphysiol.* **96**, 283.
57. Apel, K. (1979) *Eur. J. Biochem.* **97**, 183.
58. Schröder, J., Betz, B. and Hahlbrock, K. (1977) *Plant Physiol.* **60**, 440.
59. Nakakuki, M., Yamauchi, K., Hayashi, N. and Kikuchi, G. (1980) *J. Biol. Chem.* **255**, 1738.
60. Yamauchi, K., Hayashi, N. and Kikuchi, G. (1980) *J. Biol. Chem.* **255**, 1746.
61. Kumar, C. C. and Padmanaban, G. (1980) *Biochim. Biophys. Acta* **607**, 337.
62. Picket, C. B., Rosenstein, N. R. and Jeter, R. L. (1981) *Exp. Cell Res.* **132**, 225.
63. Muthukrisnan, S., Chandra, G. R. and Maxwell, E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6181.
64. Zurfluh, L. L. and Guilfoyle, T. J. (1980) *Proc. Natl Acad. Sci. U.S.A.* **77**, 357.
65. Kamalay, J. C. and Goldberg, R. B. (1980) *Cell* **19**, 935.
66. Auger, S., Baulcombe, D. and Verma, D. P. S. (1979) *Biochim. Biophys. Acta* **563**, 496.

67. Legocki, R. P. and Verma, D. P. S. (1980) *Cell* **20**, 153.
68. Silflow, C. D. and Rosenbaum, J. L. (1981) *Cell* **24**, 81.
69. Muller, M., Viro, M., Balke, C. and Kloppstech, K. (1980) *Planta* **148**, 444.
70. Link, G., Coen, D. M. and Bogorad, L. (1978) *Cell* **15**, 725.
71. Trachsel, H., Ranu, R. S. and London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3654.
72. Ochoa, S. (1979) *Naturwiss. Rundsch.* **32**, 103.
73. Trewavas, A. (1981) *Plant Cell Environ.* **4**, 203.