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PEROXIDASE SYNTHESIS IN AND MEMBRANES FROM CULTURED PEANUT CELLS

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Abstract—Peroxidase synthesis in peanut cells grown in suspension culture was only affected by incubation with actinomycin D during a brief time following the lag phase of growth. A greater effect was noted at that time using cycloheximide, with lesser effects at later phases. Incubation with chloramphenicol had no effect. These observations along with the presence of peroxidase linked to the endoplasmic reticulum, suggest synthesis of the enzyme occurs on these organelles.

INTRODUCTION

Peroxidase (donor: H_2O_2, oxidoreductase; EC 1.11.1.7.) as a model enzyme has been studied for several decades [1], particularly those on its relation to plant growth. More recently peroxidase has been used to demonstrate successful fusion of plant cells [2]. Nevertheless there remains a dearth of information on the biosynthesis of the enzyme. A detailed examination of its biosynthesis should be possible now that translation of *m*RNA for plant enzymes *in vitro* has been achieved [3, 4].

Studies on achlorophyllous peanut cells in a suspension culture containing 3% sucrose revealed the release of copious amounts of peroxidase [5]. Further investigations showed that the protoheme pool in these cells is 10 to 20 fold greater than that in leaves and that this pool could serve as a source for the heme moiety of peroxidase [6]. Peroxidase with a high specific activity has been extracted from such cells with a high salt buffer [7] and can be purified by affinity chromatography on a Concanavaline A-Sepharose gel column [8]. Using these procedures the following study attempts to elucidate the timing of biosynthesis of peroxidase and the site of its biosynthesis.

RESULTS AND DISCUSSION

The assay of peroxidase released by peanut cells in suspension medium is a convenient means of monitoring peroxidase synthesis in such cells [7]. The diminished release of peroxidase by 6 day old cells in a second and subsequent 2 hr incubation period with 0.2 mM cycloheximide agrees well with the inhibition of protein synthesis [5]. Nevertheless it would be presumptive to assume that the cells are equally responsive to inhibitors throughout their 14 day growth cycle [7].

Incubation of peanut cells in a medium containing 20 μg/ml actinomycin D failed to reduce peroxidase release from cells two days following subculture (Table 1). This result confirmed earlier data on the lack of synthesis of ribosomal RNA during the first two days of the lag phase in growth of such cells [9]. Moreover peroxidase synthesis in wheat embryos is due to conserved messenger RNA produced in the early stages of germination [10]. Mouse embryo cells also contain long lived messenger RNA that is unaffected by actinomycin D treatment [11]. There appears to be a relatively brief period in the culture of peanut cells when new messenger RNA for peroxidase is being formed (Table 1). This period (4–5 days) occurs immediately after the lag phase and corresponds to the time when overall protein synthesis in cultured peanut cells is most active [7].

Table 1. Decrease in peroxidase released at various times of cell subculture after incubation for two successive periods (2 hr) with 20 μg/ml actinomycin D

	Days of subculture				
	2	4	5	7	9
Period	% Decrease in peroxidase released				
1	0	5	2	0	0
2	4	42	39	0	0

The greatest effect on peroxidase synthesis by incubation with 0.2 mM cycloheximide occurs shortly after transferring the peanut cells into fresh medium (Table 2). But cycloheximide reduces peroxidase released at all stages observed in the culture cycle. Conversely, incubation with chloramphenicol even at 10^{-4} M had no effect on peroxidase synthesis at 3 days after subculture or any time later. Chloramphenicol at 10^{-6} M causes a severe inhibition on cell wall expansion in artichoke tissue [12]. Furthermore it was observed that either 0.2 mM cycloheximide or 10^{-4} M chloramphenicol inhibited the incorporation of ^{3}H-leucine into proteins of three day old peanut cells up to 90 and 9% respectively during the

Table 2. Decrease in peroxidase released at various times of cell subculture after incubation for three successive periods (2 hr) with 0.2 mM cycloheximide

	Days of subculture				
	3	6	9	11	14
Period	% Decrease in peroxidase released				
1	27	0	0	0	0
2	71	38	32	22	25
3	90	73	68	52	56

first 2 hr of incubation. It is during these first 2 hr that the labelling of proteins is most rapid [5]. These results suggest that the apoenzyme part of peroxidase is synthesized in the cytoplasm rather than in the mitochondria [13] and probably on the ribosomes associated with the membranes [14]. This hypothesis appears to receive support from the following deductions. Proteins released by animal cells have been shown to be formed on the rough endoplasmic reticulum and are transported through the cytoplasm to the plasmalemma by Golgi apparatus [15]. Electron microscopic observations of the peanut cells in suspension culture have revealed an abundance of Golgi apparatus and rough endoplasmic reticulum [5].

These observations led to attempts to isolate membranes from the 10000 *g* supernatant according to procedures described for their fractionation from rat cells [16]. Special attention was paid to the fractions found on the interphase of 1.3 M and 1.5 M as well as 1.5 M and 2 M sucrose which represented smooth and rough endoplasmic reticulum respectively for rat liver extract. When these fractions of peanut cell extract were assayed with 0.0025 M 2,6-dichloroindophenol and 0.1 M succinate, no significant reaction was observed compared to the assay carried out on mitochondria. This indicates that little if any contamination with peanut mitochondria occurred in these fractions. Conversely, glucose-6-phosphatase activity could readily be demonstrated (Table 3) which is typical for membranous fractions [16] and is the marker enzyme for these organelles. It was assumed that all soluble peroxidase was washed away from these membrane fractions. Therefore the membrane fractions were extracted with phosphate buffer pH 7 containing 0.8 M KCl, the extract dialyzed overnight and subsequently passed through a Concanavaline A-Sepharose gel. It was noted that the heaviest fraction, assumed to be rough endoplasmic reticulum, contained more enzyme units of peroxidase. In Table 3 this is shown with regard to the eluted fraction, which is the most pure peroxidase fraction [8]. What the significance is for this phenomenon can not yet be ascertained without studying *in vitro* synthesis of peroxidase from this isolated fraction. However, it does not alter the conclusion stated above that peroxidase is probably synthesized on rough endoplasmic reticulum. Further studies along this line are complicated because of the hemoproteinaceous nature of the peroxidase. Nevertheless, *in vitro* translation of the *m*RNA for the apoenzyme part of leghemoglobin has been accomplished [17]. A difference between leghemoglobin and peroxidase is that the synthesis of the former occurs predominantly on free ribosomes [18]. But leghemoglobin is not known to be released by plant cells and receives its heme moiety from bacteria [19]. Despite these facts, some relationship between peroxidase and leghemoglobin has been suggested [20, 21].

Another hemoprotein, cytochrome P450, is synthesized on a mitochondria–rough endoplasmic reticulum complex of rat liver [22]. It was shown that injections of rats with 60 mg/kg of cobaltous chloride caused a drastic reduction in the synthesis of the cytochrome over 2 days. Culturing peanut cells in a medium supplemented with 60 mg/l. cobaltous chloride for 4 days reduced the amount of peroxidase released at that time to 50% of control. It was noted, however, that the growth of these cells in the first week of culture was only 30% of control based on fresh weight. But the cells did not appear to contain disorganized protoplasm.

Table 3. Characteristics of fractions from 10000 *g* supernatant of peanut extract following sucrose (1.3, 1.5 and 2 M) gradient centrifugation

Interphase	Protein mg	P_i* μM	Con. A effluent E.U.†	Con. A eluant E.U.†
1.5	2	60	0.7	0.1
2.0	0.5	150	0.8	0.5

*Glucose-6-phosphatase activity expressed as μM P_i liberated/mg protein/hr.

†Enzyme Units of peroxidase = ΔA_{460}/min/11.3

EXPERIMENTAL

Peanut cells were grown in the medium of ref. [23] and transferred routinely biweekly into fresh medium [7]. At appointed times, inhibitors were added to the medium for successive 2 hr periods. Assays for either/or both, peroxidase release and protein synthesis in the cells were then carried out as reported earlier [5]. Studies on the membrane fractions were carried out as in ref. [16] following the decompression of filtered cells under N_2 [5] in 0.0375 M Tris maleate pH 6.4 containing 0.5 M sucrose, 1% dextran, 0.005 M $MgCl_2$ and 0.004 M cysteine. The homogenate was passed through miracloth and centrifuged at 10000 *g* for 10 min. From the supernatant, 37 ml was placed on a discontinuous gradient (6 ml of 2 M; 8 ml of 1.5 M and 8 ml of 1.3 M sucrose in the above buffer) and centrifuged at 90000 *g* for 90 min. The organelles were collected by pasteur pipette; the sucrose diluted 3× with buffer, and the whole centrifuged at 20000 *g* for 15 min. The pellet was extracted in 0.05 M P_i, pH 7, containing 0.8 M KCl and 0.005 M bisulfite. Following dialysis the extract was passed through a Concanavaline A-Sepharose gel and the affinity fraction eluted with 10% α-methyl-D-glucoside as reported [8]. The activity of glucose-6-phosphatase was determined by incubation with 11 mM glucose 6-phosphate in 55 mM Tris and 11 mM mercaptoethanol pH 6.6 for 1 hr [24]. Following precipitation with TCA, the liberated P_i was determined in the supernatant by the technique of ref. [25].

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REFERENCES

1. van Fleet, D. S. (1947) *Biodynamica* **6**, 125.
2. Carlson, P. S., Smith, H. H. and Dearing, R. D. (1972) *Proc. Natl. Acad. Sci. U.S.* **69**, 2292
3. Verma, D. P. S., Maclachlan, G. A., Byrne, H. and Ewing, D. (1975) *J. Biol. Chem.* **250**, 1019
4. Ragg, H., Schröder, J. and Hahlbrock, K. (1977) *Biochim. Biophys. Acta* **474**, 226
5. van Huystee, R. B. and Turcon, G. (1973) *Can. J. Botany* **51**, 1169
6. van Huystee, R. B. (1977) *Can. J. Botany* **55**, 1340.
7. Kossatz, V. C. and van Huystee, R. B. (1976) *Can. J. Botany* **54**, 2089
8. van Huystee, R. B. (1976) *Can. J. Botany* **54**, 876.
9. Verma, D. P. S. and Marcus, A. (1973) *Devel. Biol.* **30**, 104.
10. Taneja, S. R. and Sachar, R. C. (1976) *Phytochemistry* **15**, 1589.
11. Tasca, R. J. and Hillman, N. (1970) *Nature* **225**, 1022.
12. Setterfield, G. (1971) *Planta* **95**, 45.
13. Schatz, G. and Mason, T. L. (1974) *Ann. Rev. Biochem.* **43**, 51.
14. Glazer, R. J. and Sartorelli, A. C. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1418.
15. Palade, G. (1975) *Science* **189**, 347.
16. Morré, D. J. (1973) in *Molecular Techniques and Approaches in Developmental Biology* (Chrispeels, M. J. ed.) Wiley-Interscience New York.

17. Verma, D. P. S., Nash, D. T. and Schulman, H. M. (1974) *Nature* **251**, 74.
18. Verma, D. P. S. and Bal, A. K. (1976) *Proc. Natl. Acad. Sci. U.S.* **73**, 3843.
19. Cutting, J. A. and Schulman, H. M. (1971) *Biochim. Biophys. Acta* **229**, 58.
20. Moustafa, E. (1963) *Nature* **199**, 1189.
21. Appleby, C. A. (1974) in *Biology of Nitrogen Fixation* (Quispel ed.) Chap. 11.5. North Holland, Amsterdam.
22. Correia, M. A. and Meyer, U. A. (1975) *Proc. Natl. Acad. Sci. U.S.* **72**, 400.
23. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Plantarum* **18**, 100.
24. Morré, D. J. (1971) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds). Academic Press, New York.
25. Fiske, C. H. and Subbarow, Y. (1956) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds). Academic Press, New York.