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SITE OF HAEM SYNTHESIS IN CULTURED PEANUT CELLS

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Key Word Index—*Arachis hypogaea*; Leguminosae; peanut; cell suspension culture; cationic peroxidase; haem; δ-aminolevulinic acid; mitochondria; amyloplast; medium; haemoprotein.

Abstract—Cationic peroxidase is the major haemoprotein secreted by cultured peanut cells, into their medium, as determined by immunoprecipitation. The haem moiety of cationic peroxidase has been identified as protohaem, based on results obtained by mass spectrometry. By incubating the cultured cells with [^{14}C]-δ-aminolevulinic acid and subsequently isolating the mitochondria and amyloplasts from these cells, it has been shown that mitochondria are the site of synthesis of haem in these cells.

INTRODUCTION

The haem moiety for the cationic peroxidase in cultured peanut cells, was shown to be derived from glutamic acid [1]. Results verifying the derivation of haem from glutamic acid in a red alga were reported later by Weinstein and Beale [2]. However, in the case of the haem moiety for cationic peanut peroxidase, the site of synthesis in terms of the specific organelle is yet unknown. Cultured peanut cells are achlorophyllous because they are grown in high sucrose medium [3]. It may be questioned whether the synthesis of haem destined for cationic peroxidase is a mere diversion of the porphyrin pathway in plastids (amyloplasts in these cells) from chlorophyll to haem synthesis under these culture conditions [4]. Especially, since cationic peroxidase forms two percent of the total protein synthesis in these cells [5] as compared to 0.2% in peanut leaves [6]. However, cationic peroxidase from cultured cells remains immunologically related to that in peanut leaves [6]. This elevated synthesis of cationic peroxidase and its selective release into the medium [4, 7] has enabled us to purify it, in a relatively few steps [8]. This homogeneous preparation of cationic peroxidase has been used to raise antibodies in rabbits and then to use the antibodies for immunoprecipitation of peroxidase from culture medium and cell extracts [1, 6].

In the present study through incorporating [^{14}C]-δ-aminolevulinic acid (ALA) into cultured peanut cells and immunoprecipitating haem-labelled cationic peroxidase from the medium, it has been shown, that it is the major haemoprotein released into the medium of cultured peanut cells. Using the same cells, incubated with [^{14}C]-ALA and isolating the amyloplast and mitochondria, it has been shown that the mitochondria are the site of haem synthesis in these cells. Based on mass spectrometry, the haem in the cationic peroxidase has been identified as protohaem.

RESULTS AND DISCUSSION

The results presented in Table 1 show that most of the ALA taken up by the cultured peanut cells is incorporated into the haem, as compared to the protein moiety.

Table 1. Incorporation of ALA into the haem moiety of peanut peroxidase

Fraction	Vol. (ml)	Immunoprecipitate (dpm × 10^3)	Apoprotein (dpm × 10^3)	Haem (dpm × 10^3)
Medium	12	29.3	1.9	26.3
177 000 *g* Supernatant	20	28.5	1.6	25.2

Fifty ml of enriched cell cultures (3 days old *ca* 15–20 g fr. wt of cells) were incubated with 1.1 MBq of [^{14}C]-ALA for 4 hr in the light. The cells and medium were separated by filtration, under vacuum. The cells were washed with 200 ml of cold distilled water to remove any adsorbed radioactivity. The cells were subjected to differential centrifugation as described in Experimental to obtain 177 000 *g* supernatant. Cationic peroxidase from 1 ml of medium and supernatant of 177 000 *g* was immunoprecipitated using antibodies against cationic peroxidase [6]. The pellet of immunoprecipitate of peroxidase was cleaved into haem and apoprotein by the cold acidified acetone method [1]. The haem in ether was dried and the radioactivity was measured in both haem and apoprotein. The data of three replicates were then calculated and the data corresponding to total volumes was calculated.

Cationic peroxidase, a haemoprotein, was immunoprecipitated from the medium of cultured cells, incubated with [^{14}C]-ALA, or from the 177 000 *g* supernatant of the cell extract. Subsequently it was cleaved into haem and apoprotein moieties. In excess of 90% of the radioactivity was observed in the haem moiety, as compared to the apoprotein (Table 1). This is in comformity with the earlier reports [9, 10] where ALA is considered to be the first committed precursor of the porphyrin pathway. However, Ellis and Greenwald [11] reported that in the green alga, *Golenkinia*, ALA may also serve as an alternate nitrogen source after its metabolism to CO_2, amino acids and dicarboxylic acids. In the present case, this does not appear to be so, since less than 10% of the [^{14}C]-ALA is incorporated into the apoprotein, as compared to the haem moiety of a specific haemoprotein, the cationic peroxidase. The sensitivity and specificity of the technique using cold acidified acetone [12] to separate haem and apoprotein moieties of cationic peroxidase has been discussed earlier [1].

The results presented in Table 2 show that the immunoprecipated cationic peroxidase contained nearly 90% of the radioactivity that was incorporated into the total medium proteins precipitated by TCA. Since the cells were incubated with [^{14}C]-ALA, which is incorporated into the haem moiety of proteins, it suggests that cationic peroxidase is the major haemoprotein in the medium. This is in agreement with the results reported earlier [13], where in the gels stained for haemoproteins from the medium of cultured peanut cells, cationic peroxidase formed the major protein band. Regarding the 177 000 *g* supernatant, the haemoproteins formed only 20% of total synthesized proteins (Table 2). This may be due to the dilution of peroxidase with other haemoproteins that occur also in the soluble phase, isolated in 177 000 *g* supernatant. In the microsomal fraction, cationic peroxidase formed only 70% of the total haemoproteins (Table 2). Microsomes usually represent the compartment that contains proteins destined for export [14, 15]. Cationic peroxidase is the major haemoprotein in the medium, and this peroxidase fraction represents nearly 75% of all peroxidase activity in the medium [8].

The results presented in Table 3 are based on organelles isolated from [^{14}C]-ALA labelled cells. They show that about 15-fold greater radioactivity was derived from mitochondria than from amyloplasts. In fact, in amyloplasts the radioactivity detected was so low that it may be regarded to be near background (Table 3). This part of the study suggested that mitochondria are the major site of haem synthesis in cultured peanut cells. It would negate the notion that the increased peroxidase synthesis in cultured peanut cells is a diversion of porphyrin pathway from chlorophyll to haem synthesis. Mitochondria do contain various endogenous haemoproteins (e.g. cytochromes) whose haem moiety might have been responsible for the radioactivity observed in this organelle but whose synthesis does not occur in this organelle. The slow turnover rate (1300 hr) of the cytochromes present in rat liver [10] rules out the possibility that they were synthesized in significant amounts because cultured cells were incubated with [^{14}C]-ALA for only 4 hr. Conversely, cultured peanut cells contain a endogenous haem pool [16] which necessitated at least a 4 hr period of incubation in order to have a significant proportion labelled. These results further confirmed the earlier suggestion based on circumstantial evidence [16] that mitochondria instead of amyloplasts are the site of haem synthesis in cultured peanut cells. Moreover, amyloplasts are laden with starch and have a relatively very low protein content (Table 3) as compared to mitochondria.

The haem moiety of the cationic peroxidase ($R_z > 3$) was cleaved, purified and its mass-ion spectrum determined. The major ion of the isolated haem was observed at m/z 616, corresponding to the M_r of protohaem [17]. The other peaks with slightly more than 50% intensity were observed at m/z 565 and 555, which may be due to the loss of a pyrrole ring or iron, respectively. This confirms that the haem moiety of cationic peroxidase is protohaem. Protohaem is know to be synthesized in both chloroplasts and mitochondria, in contrast to haem 'a' which can only be synthesized in plastids [2].

In conclusion, the evidence presented in this communication shows that protohaem destined for cationic peroxidase is synthesized in the mitochondria. This leads us to consider the interesting possibility that the synthesis of peroxidase may be under the control of mitochondria. The control of apoprotein biosynthesis in the case of catalase has been shown to be under the control of haem [18]. Further work on the depletion of haem from the cultured cells and its influence on peroxidase synthesis is under investigation.

EXPERIMENTAL

Peanut (*Arachis hypogaea* L. var 56R) cell suspension cultures derived from cotyledonary tissues were maintained routinely in a biweekly culture [19].

Isolation of organelles. The organelles (amyloplast, mitochon-

Table 2. Percentage of peroxidase to total haemoproteins in various fractions of cultured peanut cells

Fraction	Total volume (ml)	Peroxidase (immunoprecipitate) (dpm × 10^3)	Proteins (TCA precipitate)	Percent* peroxidase to haemoproteins
Medium	12	29.3	32.4	90
Supernatant 177 000 *g*	20	28.5	132.9	21
Microsomes	2	6.2	8.9	70

Cells were incubated with [^{14}C]-ALA and fractions obtained as described in Table 1. The peroxidase was immunoprecipitated as described [6], but total haemoproteins labelled with [^{14}C]-ALA were precipitated with 10% TCA and radioactivity measured.

*$\frac{\text{dpm in immunoprecipitate}}{\text{dpm in TCA precipitate}} \times 100$.

Table 3. Site of haem synthesis in cultured peanut cells

Organelle	dpm × 10^3 in haem derived from [^{14}C]-ALA	Total protein (μg)
Amyloplast	2.3	202
Mitochondria	33.2	1738

Cells were incubated with [^{14}C]-ALA as described in Table 1. Amyloplasts and mitochondria were isolated as described under Experimental. Haem from these two organelles was extracted sequentially, three times by cold acidified acetone method [1]. The haem in ether was dried and radioactivity measured.

dria and microsomes) were isolated from cultured peanut cells as described [20]. This procedure essentially consisted of homogenizing the cells in 300 mM mannitol, 5 mM EDTA, 0.05% cysteine and 30 mM MOPS, pH 7.5 and subjecting the filtrate from Miracloth to differential centrifugation. Crude amyloplasts were obtained in the 2500 *g* pellet and mitochondria were pelleted 100 000 *g*, after discarding the 10 000 *g* pellet, which contained the nuclei. Microsomes were then pelleted from 100 000 *g* supernatant at 177 000 *g* for 60 min. The amyloplasts were then purified by the method of rapid isolation of chloroplasts [21]. Mitochondria were purified by the method of ref. [22]. Following pelleting, microsomes were once again resuspended in the homogenizing buffer and pelleted as above. The homogeneity of the organelles so obtained was checked using marker enzymes and electron microscopy as described [20]. Haem was isolated from organelles and immunoprecipated cationic peroxidase [6] as described [1]. Protein was measured by the method of ref. [23].

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REFERENCES

1. Chibbar, R. N. and van Huystee, R. B. (1983) *Phytochemistry* **22**, 1721.
2. Weinstein, J. D. and Beale, S. I. (1984) *Plant Physiol.* **74**, 146.
3. van Huystee, R. B. (1977) *Acta Hortic.* **78**, 83.
4. van Huystee, R. B. and Turcon, G. (1973) *Can. J. Botany* **51**, 1169.
5. Stephan, D. and van Huystee, R. B. (1980) *Can. J. Biochem.* **58**, 715.
6. Chibbar, R. N. and van Huystee, R. B. (1983) *Z. Pflanzenphysiol.* **109**, 191.
7. van Huystee, R. B. and Lobarzewski, J. (1982) *Plant Sci. Letters* **27**, 59.
8. Maldonado, B. A. and van Huystee, R. B. (1980) *Can. J. Botany* **58**, 2280.
9. Beale, S. I. (1978) *Ann. Rev. Plant Physiol.* **29**, 95.
10. Granick, S. and Beale, S. I. (1978) in *Advances in Enzymology* (Meister, A., ed.) Vol. 46, p. 33. Wiley, New York.
11. Ellis, R. and Greenwald, M. (1985) *Plant Sci. Letters* **37**, 213.
12. Stillman, L. C. and Gassman, M. L. (1978) *Analyt. Biochem.* **91**, 166.
13. Chibbar, R. N., Cella, R. and van Huystee, R. B. (1984) *Can. J. Biochem.* **62**, 1046.
14. Hall, J. L., Flowers, T. J. and Roberts, R. M. (1982) in *Plant Cell Structure and Metabolism.* 2nd edn, p. 213. Longman, London.
15. Palade, G. (1975) *Science* **189**, 347.
16. van Huystee, R. B. (1977) *Z. Pflanzenphysiol.* **84**, 427.
17. Stecher, P. G. Windholz, M., Leahy, D. S. (eds) (1968) *The Merck Index—An Encyclopedia of Chemicals and Drugs*, 8th edn. Merck, Rahway, NJ.
18. Hamilton, B., Hofbauer, R. and Ruis, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7609.
19. Kossatz, V. C. and van Huystee, R. B. (1976) *Can. J. Botany* **54**, 2089.
20. Chibbar, R. N. and van Huystee, R. B. (1986) *J. Plant. Physiol.* (submitted).
21. Mills, W. R. and Joy, K. W. (1980) *Planta* **148**, 75.
22. Bonner, W. D., Jr. (1976) *Methods Enzymol.* **10**, 126.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.