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GLUTAMIC ACID IS THE HAEM PRECURSOR FOR PEROXIDASE SYNTHESIZED BY PEANUT CELLS IN SUSPENSION CULTURE

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Key Word Index—*Arachis hypogaea*; Leguminosae; cell suspension cultures; medium; cationic peroxidase; haem; glutamic acid; glycine; immunoprecipitation.

Abstract—The major cationic fraction of peroxidase forms one-sixth of all the proteins released by cultured peanut cells into their growth medium. Thus, the spent medium is an ideal source of this peroxidase fraction. By incubating peanut cells in the presence of either [^{14}C]glutamic acid or glycine it has been shown that the haem moiety of the major cationic peroxidase isolated from the medium, is derived from glutamic acid.

INTRODUCTION

It has been acknowledged for quite sometime that δ-aminolevulinic acid (ALA) is the precursor for both chlorophylls and haem [1]. But it is only during the last decade [2] that it was shown that the ALA destined for chlorophyll is derived from a C_5 precursor, i.e. glutamic acid, α-ketoglutarate or glutamine, instead of glycine and succinyl Co-A as is the case for the haem moiety of haemoglobin [1, 3]. Whereas the ultimate destination of the ALA derived from the C_5 precursors into chlorophyll is well documented in the literature [4], its role in the synthesis of haem in plants is not clear. From indirect evidence it has been suggested that perhaps haem in plants may also be derived from glutamate [5, 6]. Yet so far no specific haem protein in plants has been studied in terms of the synthesis of its haem moiety.

Peroxidase (EC 1.11.1.7), a haemoprotein, accounts for 2% of the total protein synthesized by cultured peanut cells [7] and is selectively released into the suspension medium [8]. The major cationic fraction of this peroxidase from the medium can be easily purified (*RZ* value > 3) to a major single protein band by SDS gel electrophoresis [9] and antibodies have been raised against this fraction. With the aid of immunoprecipitation, it has been possible to study the transport and release of peroxidase by cells in suspension cultures [10]. By employing this technique of immunoprecipitation, a study on the fate of glycine and glutamic acid as potential precursors for the haem moiety of the major cationic fraction of peanut peroxidase has been undertaken.

RESULTS AND DISCUSSION

The data in Table 1 confirmed that the medium was indeed a rich source of peroxidase as suggested on circumstantial evidence [9]. Compared to the 2% of total intracellular proteins [7], it was clear that the selective secretion of peroxidase occurs [8]. Therefore, the peroxidase from the medium was well suited for a study of the incorporation of either glycine or glutamic acid into the haem moiety of this peroxidase.

Before carrying out the actual experiment, it was necessary to demonstrate the successful cleavage of the haemoprotein. This was imperative in view of the fact, that incorporation of amino acids proceeds not only into haem, but more so into the apoprotein moiety. It was found that the modified cold acetone method [11] resulted in the recovery of a clean haem moiety as

Table 1. Comparison of peroxidase to total proteins in the medium of peanut cells in suspension culture

Labelled amino acid	Vol. of medium (ml)	Radioactivity (10^{-3} × cpm)		% of peroxidase to total proteins
		Total proteins	Peroxidase	
[^{35}S]Methionine	8.0	235	39.4	16
[U-^{14}C]Leucine	6.0	29.8	4.6	15

Aliquots (10 ml) of enriched cell suspension culture (4 days old *ca* 2 g fr. wt of cells) were incubated with the above labelled amino acids for 2 hr in the light. The medium was separated from the cells by filtration and aliquots (2 ml for total proteins and 0.5 ml for peroxidase) were taken to precipitate total proteins with 10% TCA and peroxidase by immunoprecipitation.

measured by its reduced minus oxidized pyridine haemochrome spectrum (Fig. 1). In addition, incubation of cells with [^{35}S]methionine resulted in a radioactive immunoprecipitated pellet, but no radioactivity could be detected in the isolated haem fraction as was to be expected.

In the classical pathway of ALA synthesis, during the condensation of glycine and succinyl CoA to form ALA, the C-1 position of glycine is decarboxylated [1, 12], it was only natural to use [1-^{14}C]glycine as a control for [2-^{14}C]glycine incorporation. However, as the data in Table 2 shows, there was virtually no radioactivity associated with the haem fraction no matter whether glycine was labelled at C-1 or C-2. This provided a direct indication that glycine was not the precursor for the haem moiety in this case. It should be noted here, that there were no obvious hindrances to the uptake of glycine by the cells, as seen by the incorporation of glycine into the apoprotein moiety of the immunoprecipitated protein (Table 2). Moreover, if the cells were incubated with [U-^{14}C]glutamic acid, it was shown that even though less of the amino acid as compared to glycine was incorporated into the apoprotein moiety, more of it was detected in the haem moiety as compared to glycine (Table 2). The free amino acid pool of glycine is half that of glutamic acid in these cultured cells [13]. This, together with the slightly higher molar ratio of glycine than glutamic acid in the peroxidase [14], explains the higher incorporation of glycine into the apoprotein moiety. However, the higher incorporation of glutamic acid into the haem moiety, in spite of its relatively larger free pool in the cells, further strengthens the results and conclusions drawn. Thus, by a comparatively direct and precise approach using an immunoprecipitated protein further support has been provided for the earlier hypothesis that the haem in plants is also derived from glutamic acid, a C_5 compound [5, 6].

Finally, it may be added that a 6 hr incubation period was used for this experiment, since the earlier studies had indicated the presence of a considerable haem pool in cultured peanut cells [15]. However, the consideration that glutamic acid may be deaminated and transformed to succinyl Co-A and as such may enter the porphyrin pathway, was rejected on the basis that [2-^{14}C]glycine was not incorporated. Moreover, twice the amount of radioactivity was supplied for the latter (Table 2). The question still remains as to the site of haem synthesis in plant cells [16]. It was shown earlier [17] that more porphyrins could be detected in the mitochondria than in the plastids of cultured peanut cells. Further work on this is under consideration.

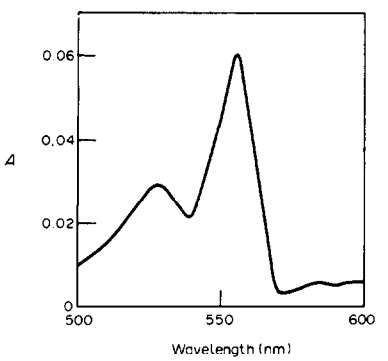

Fig. 1. Reduced minus oxidized pyridine haemochrome spectrum of haem extracted from the immunoprecipitate of the major cationic peroxidase. The immunoprecipitated peroxidase was cleaved into the apoprotein and haem moieties. The haem was dried under N_2 and dissolved in alkaline pyridine and divided equally between two cuvettes. In the sample cuvette $Na_2S_2O_4$ was added to reduce the haem and the spectrum was recorded.

EXPERIMENTAL

Peanut (*Arachis hypogaea* L. var. Virginia 56R) cell suspension cultures derived from cotyledonary tissue were maintained routinely [18].

Separation of the haem and apoprotein moiety. The peroxidase immunoprecipitate was obtained from the respective mediums (0.5 ml) and washed twice to remove any adhered radioactivity as described [10]. This pellet was treated with 0.5 ml cold acidified (2% v/v conc. HCl) Me_2CO as described [11]. Following a second extraction, the top organic phases separated by centrifuging at 25 000 *g* for 10 min at 4° were combined. To this was then added 0.5 vol. chilled peroxide free Et_2O and 1.5 vol. of chilled H_2O. The haem in the top organic phase was transferred after

Table 2. Incorporation of glycine or glutamic acid into the haem and apoprotein moiety of the peroxidase recovered from the medium

Labelled amino acid	Radioactivity (cpm/2 ml of medium)		% cpm in haem to that in apoprotein moiety
	Apoprotein	Haem	
[1-^{14}C]Glycine (25 μCi)	9,056	7	0.07
[2-^{14}C]Glycine (50 μCi)	19,165	24	0.12
[U-^{14}C]Glutamic acid (25 μCi)	4,868	255	5.20

Aliquots (10 ml) of enriched cell suspension culture (3 days old; *ca* 2 g fr. wt of cells) were incubated with the above labelled-precursors for 6 hr in the light. The medium was separated by filtration and four aliquots of 0.5 ml each from each treatment were used for immunoprecipitation. The pellet of peroxidase immunoprecipitate was cleaved into the haem and apoprotein moiety, and the haems (in ether) from the four replicates of each treatment, were pooled together for the respective treatments to give cpm/2 ml of medium. Similarly, the apoprotein moieties from each treatment were also pooled to give corresponding data for 2 ml of medium.

centrifugation at 3000 *g* for 5 min at 4° to a scintillation vial and dried. Both the dried haem as well as the apoprotein were separately dissolved in Aquasol and radioactivity was measured.

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REFERENCES

1. Granick, S. and Beale, S. I. (1978) in *Advances in Enzymology* (Meister, A., ed.) Vol. 46, p. 33. J. Wiley, New York.
2. Beale, S. I. and Castelfranco, P. A. (1974) *Plant Physiol.* **53**, 297.
3. Gibson, K. D., Laver, W. G. and Neuberger, A. (1958) *Biochem. J.* **70**, 71.
4. Castelfranco, P. A. and Beale, S. I. (1981) *Chlorophyll Biosynthesis in The Biochemistry of Plants* (Hatch, M. D. and Boardman, N. K., eds.) a comprehensive treatise, Vol. 8, pp. 376–423. Academic Press, New York.
5. Castelfranco, P. A. and Jones, O. T. G. (1975) *Plant Physiol.* **55**, 485.
6. Troxler, R. F. and Offner, G. D. (1979) *Arch. Biochem. Biophys.* **195**, 53.
7. Stephan, D. and van Huystee, R. B. (1981) *Z. Pflanzenphysiol.* **101**, 313.
8. van Huystee, R. B. and Turcon, G. (1973) *Can. J. Botany* **51**, 1169.
9. Maldonado, B. A. and van Huystee, R. B. (1980) *Can. J. Botany* **58**, 2280.
10. van Huystee, R. B. and Lobarzewski, J. (1982) *Plant Sci. Letters* **27**, 59.
11. Stillman, L. C. and Gassman, M. L. (1978) *Analyt. Biochem.* **91**, 166.
12. Akthar, M., Abboud, M. M., Barnard, G., Jordan, P. and Zaman, Z. (1976) *Philos. Trans. R Soc. London Ser. B.* **273**, 117.
13. Verma, D. P. S. and van Huystee, R. B. (1970) *Can. J. Biochem.* **48**, 444.
14. van Huystee, R. B. and Maldonado, B. (1982) *Physiol. Plant.* **54**, 88.
15. van Huystee, R. B. (1977a) *Can. J. Botany* **55**, 1340.
16. van Huystee, R. B. (1977b) *Z. Pflanzenphysiol.* **84**, 427.
17. van Huystee, R. B. (1977c) *Acta Hortic.* **78**, 83.
18. Kossatz, V. C. and van Huystee, R. B. (1976) *Can. J. Botany* **54**, 2089.