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BIOSYNTHESIS OF PEROXIDASE IN PEANUT CELLS IN SUSPENSION CULTURE

by

Ravindra Nath Chibbar

Department of Plant Sciences

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

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ABSTRACT

Peroxidase (E.C. 1.11.1.7) has been widely used as a marker of altered growth and development in plants. However, a definitive role of physiological significance has yet to be ascribed. This may be partially due to studies that have been performed with a peroxidase molecule neither isolated to purity nor characterized from the system of investigation. A cationic peroxidase (molecular weight 40 kD) had been purified to apparent homogeneity and characterized from peanut cells in suspension culture. Antibodies were raised against this protein molecule in rabbits and used for monitoring biosynthetic studies on the peroxidase molecule in peanut cells in culture.

The cationic peroxidase constituted one sixth of the total proteins released in the medium of cultured peanut cells. Peanut plant leaves also secreted the same peroxidase into their intercellular spaces as determined by immunodiffusion assays. Peanut cells in culture, however, synthesized ten fold more peroxidase than peanut leaves.

The technique of differential centrifugation to isolate cell organelles (amyloplasts, nuclei, mitochondria and microsomes) was used in conjunction with immunoprecipitation to localize peroxidase during its biosynthesis in cultured peanut cells. Most of the newly synthesized peroxidase was found to be associated with the microsomal fraction. This peroxidase from the microsomal fraction was only released when microsomes were treated with high salt buffer (phosphate buffer 0.05 M with 0.8 M potassium chloride). This indicated that the

intracellular origin of high ionic extract of peroxidase was associated with the microsomal pellet. IgGs against peroxidase were purified by immunoaffinity chromatography and were used to precipitate peroxidase synthesizing polysomes from cultured peanut cells. It was shown that most of the newly synthesized peroxidase was associated with membrane bound polysomes.

The heme moiety of peroxidase was demonstrated to be synthesized from glutamic acid and not from glycine and succinyl CoA, as is the case for heme synthesis in animals. The heme moiety was identified as protoheme, based on mass spectrometry. It was also determined that the heme was synthesized in mitochondria of cultured peanut cells. The heme moiety appeared to be present in equimolar concentration to apoprotein, in peroxidase. The dissociation of the heme moiety from the apoprotein, did not significantly alter the size or shape of the molecule as determined by SDS-PAGE, analytical centrifugation and immunodiffusion assays. The heme moiety was found to be essential for peroxidase as well as IAA-oxidase activities of this molecule.

An anionic fraction of peroxidase was also purified from the medium of cultured peanut cells. No appreciable differences in the enzymatic activities associated with peroxidase could be detected, between these two molecules, as reported in literature. The cationic form of peroxidase was the major form that was secreted by peanut cells in culture. The significance of the secretion of cationic form in relation to its mode of action is discussed.

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LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
Bq	Becquerel (1 disintegration per second)
CMC	Carboxy methyl cellulose
CMS	Carboxy methyl sephadex
CNBr	Cyanogen-bromide
cpm	counts per minute
DCP	2,4 dichlorophenol
DEAE	Diethyl amino ethyl
DOC	Deoxycholic acid
dpm	disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetate
EGTA	Ethylene glycol-bis (2-aminoethyl ether) N,N'-tetracetic acid
EU	Enzyme units
HRP	Horse radish peroxidase
IAA	Indole-3-acetic acid
IDP	Inosine 5'-diphosphate
IgG	Immunoglobulin G
kD	Kilo-dalton
L.A.	Levulinic acid
MES	2-(N-morpholino) ethane sulphonic acid
MOPS	3-(N-morpholino) propane sulphonic acid

PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
PVP	Polyvinylpyrrolidone
R_m	Relative electrophoretic mobility
rpm	Revolutions per minute
RZ	Reinheitzahl value (A_{407nm}/A_{280nm})
S	Svedberg unit (10^{-13} seconds)
SA	Specific activity
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SE	Standard error
TCA	Trichloroacetic acid
TEMED	N',N',N',N'-tetramethylene diamine
TMBZ	3,3',5,5'-tetramethyl-benzidine
Tricine	N[tris(hydroxymethyl) methyl] glycine
Tris	tris-(hydroxymethyl)-amino methane

CHAPTER 1

INTRODUCTION

1.1 General

An understanding of processes underlying growth and differentiation still remains a challenging problem. Growth may be defined as an irreversible change in size of a cell, organ or whole organism and is associated with qualitative and quantitative changes. The study of this phenomenon, for example in higher plants, is much more complex, than would be assumed from a peripheral observation. In animals growth occurs throughout the organism, in contrast to higher plants, where growth is restricted to localized embryonic regions. The basic definition of growth of plant cells has led to the utilization of plant tissue culture, in an attempt to unravel the mysteries of plant growth and development. Nevertheless, both for tissue culture and *in vivo* studies of plants, the identification of specific markers, which could be associated with specific stages of growth and differentiation is required.

1.2 Peroxidase as biochemical marker of growth and differentiation

Isoenzymes (multiple molecular forms) have been used as biochemical markers in a number of biological investigations (see references cited in Tanksley and Orton, 1983; Brewbaker, 1984). Amongst the various enzymes used in plants, peroxidase (E.C.1.11.1.7) has been most

frequently employed both for <u>in vivo</u> and <u>in vitro</u> cultures (Scandalios and Sorenson, 1977; Wetter and Dyck, 1983). The obvious reason is the involvement of peroxidase in the metabolism of indole-3-acetic acid (IAA) (Scandalios and Sorenson, 1977; Sembdner <u>et al</u>., 1980; Grambow and Langenbeck-Schwich, 1983). Indole-3-acetic acid is the ubiquitious natural plant growth regulator and plays a major role in plant growth and development (Gamburg, 1982; Cleland, 1983; Trewavas, 1983). Besides, the consideration mentioned above, another reason is the ubiquity of peroxidase and the ease of its assay (van Huystee and Cairns, 1980, 1982). For these reasons, other workers such as geneticists and systematicists have also used it as a phylogenetic marker (Reynolds, 1979; Gottlieb, 1981; Houston and Hood, 1982; Moore and Collins, 1982; Santamour (Jr.), 1982, 1983). In addition, plant pathologists have implicated it in the host-pathogen interactions (Fric, 1976; Hammerschmidt <u>et al</u>., 1982). Diverse functions have also been attributed to this enzyme. These include the oxidation of IAA (Sembdner <u>et al</u>., 1980; Grambow and Langenbeck-Schwich, 1983), the oxidation of phenols and their polymerization to lignin (Stafford, 1960; Gibson and Liu, 1981; Deloire and Hebant, 1982), and more recently, the oxidation of pigments (Huff, 1982; Matile and Martinoia, 1982). However, no decisive role for peroxidase of physiological importance has been demonstrated.

Most of the studies on peroxidases are concerned with either the specific activity (S.A.) in crude cell extracts using different substrates (Siegel <u>et al</u>., 1982; Boyer <u>et al</u>., 1983; Goldberg <u>et al</u>., 1983; Klisurska and Dencheva, 1983) or the concomitant changes in isoenzymes (Chibbar <u>et al</u>., 1980; Bredemeijer and Blaas, 1983; de

Forchetti and Tigier, 1983) with growth alterations. The number of peroxidase isoenzymes varies from a mere one (Nash and Davies, 1975; Srivastava and van Huystee, 1977b) to more than three dozen (Hoyle, 1978; Thomas and Delincee, 1979). These reports of high numbers of peroxidase isoenzymes have led people to question whether so many isoenzymes could actually have a function in the cell, or whether these so-called isoenzymes are artifacts (Nash and Davies, 1975; van Huystee and Cairns, 1980, 1982). Shaw (1969) as well as the International Union of Biochemistry classified isoenzymes into two major categories (Enzyme Nomenclature, 1972):

(a) distinct molecules (polypeptides) which are presumably encoded at different genetic loci;

(b) secondary modifications in the structure of a single polypeptide species which in many cases may be *in vitro* artifacts and may then be termed as pseudo-enzymes (Tsai *et al*., 1983).

It appears more likely that the peroxidase isoenzymes reported in the literature may be of the second category. It was shown by Srivastava and van Huystee (1977b) that one major anionic isoenzyme may form various so-called isoenzymes by the reaction with phenolics. A similar point was also made by Liu (1971) who could generate multiple forms of peroxidase isoenzymes by changing either the pH of the extraction medium or by other preparative procedures.

Thus, in spite of the numerous reports in literature (see references cited in Gaspar *et al*., 1982) no definitive role for peroxidase in plants has yet been postulated. Although it seems to be involved in as diverse processes as sex change in flowers (Kahlem, 1976) and salt tolerance (Siegel *et al*., 1982). The reason for this anomaly

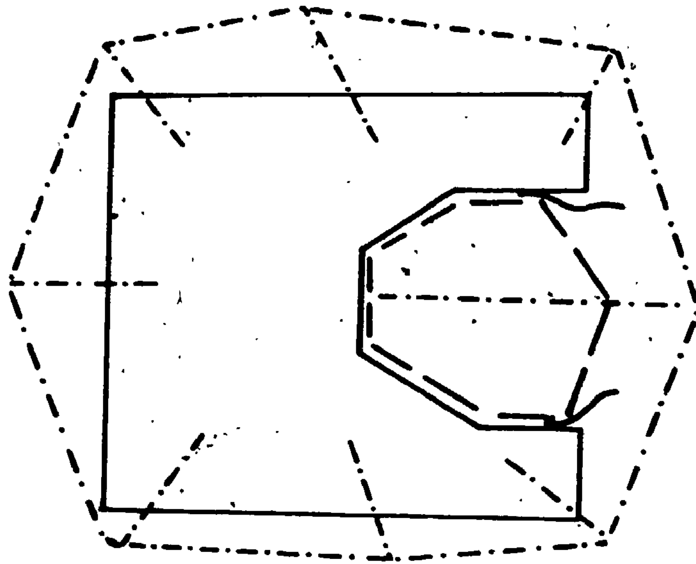
with regards to peroxidase is that most of the work has been done with crude plant/cell extracts and very little attention has been paid to the purification of peroxidase to protein level except in few cases such as tomato (Kokkinakis and Brooks, 1979).

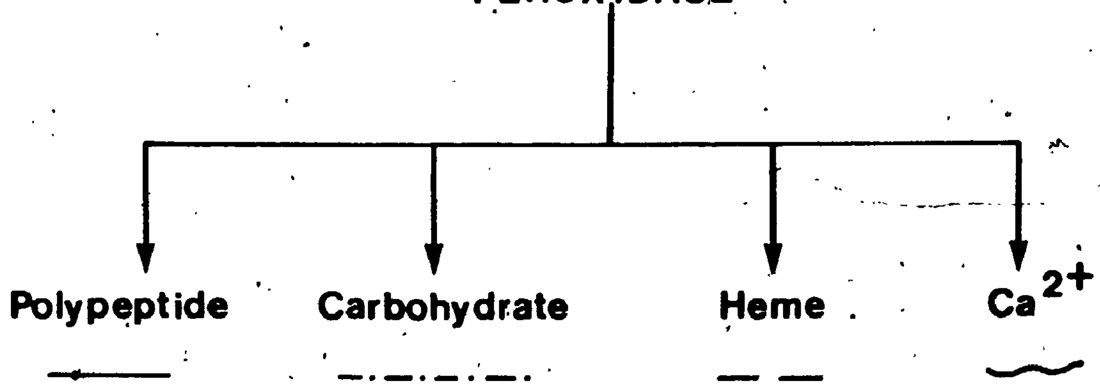
1.3 Structure of peroxidase molecule

Most of the structural aspects of peroxidase molecule have been elucidated using horseradish peroxidase (HRP) isoenzymes (Ramshaw, 1982). The number of HRP isoenzymes reported in literature varies with the technique used (Hoyle 1978). A cationic isoenzyme of HRP, identified as 'c' is the most abundant and accounts for fifty percent of peroxidase activity in the horseradish roots (Selvendran and O'Neill, 1982). This molecule is a hemoglycoprotein (Fig. 1) and so are the other peroxidases. The molecular weight for HRP isoenzyme 'c' is 44 kilodalton (kD) and it varies for other isoenzymes between 40 to 44 kD. The HRP isoenzyme 'c' consists of a hemin prosthetic group, with two Ca^{++} and 308 amino acid residues including four disulfide bridges in a single polypeptide chain, that carries eight neutral carbohydrate side chains (Welinder, 1976, 1979). The eight carbohydrate units varying in molecular weights from 1,600 to 3,000 account for approximately twenty percent of the total molecular weight (Clarke and Shannon, 1976).

The other peroxidase isoenzyme, whose structure has been studied is derived from turnip and termed as P_7 (Mazza and Welinder, 1980a,b). This isoenzyme is also basic (cationic) in nature. It has essentially the same structural arrangment as HRP isoenzyme 'c'. However, only 49 percent of the amino acid sequences are identical to HRP isoenzyme 'c'.

Figure 1. Schematic diagram (proportions of each component may vary) to illustrate the structure of peroxidase.




PEROXIDASE
Polypeptide
Carbohydrate
Heme
Ca2+

Braithwaite (1976) obtained the crystals of HRP isoenzyme 'c' by the equilibrium dialysis of Tris-buffered magnesium sulfate (2.61 M) at pH 7.7. The light brown crystals so formed were square based pyramids measuring up to 180 μm from cap to cap. The unit cell of the crystal was almost tetragonal with a volume of 3.48 x 10^6 $Å^3$ and axial lengths were a = b = 96.8 (6) Å, c = 371 (2) Å and $\alpha = \beta = \gamma = 90°$.

1.4 Heme moiety of peroxidases

The heme moiety of peroxidases is of significance because the enzymatic activities attributed to peroxidases are partially due to the electronic structure of iron in the heme moiety. The heme moiety of HRP, cytochrome c peroxidase and chloroperoxidase have been identified as ferriprotoporphyrin IX, mainly based on spectrophotometric studies (Yamazaki, 1974). Equimolar ratio between the heme and apoprotein are established for cytochrome 'c' peroxidase by reconstitution experiments (Yonetani, 1967). This homology has been used for peroxidases in general to postulate an equimolar ratio of heme and apoprotein (Scandalios 1974). No studies on the identification of heme have been done in other peroxidases investigated. There is enough evidence in animal systems which indicate that the heme prosthetic group may be different in peroxidases from different sources. The peroxidase from thyroid (Krinsky and Alexander, 1971; Hosoya and Morrison, 1967) as well from milk (Hultquist and Morrison, 1965) contain protoheme as the prosthetic group, while myeloperoxidase has heme 'a' as the prosthetic group (Schultz and Shmuckler, 1964; Newton et al., 1965).

1.5 Biosynthetic studies.

The structure of HRP isoenzyme 'c' is fairly well known, but the studies on biosynthesis of this peroxidase or any other isoenzyme are lacking. Studies with inference on biosynthesis have been reported from other unidentified peroxidases using metabolic inhibitors and assay of peroxidase enzyme activity. Actinomycin 'D' an inhibitor of transcription was found to reduce the peroxidase activity, which normally increases following excision in sugarcane stalk tissue (Gayler and Glasziou, 1968) and in lentil embryonic axis (Khan et al., 1972). In cultured peanut cells, the inhibitory effect of Actinomycin D occurs only for a brief period following the lag phase of growth (van Huystee, 1978). But there are several reports showing either no effect in wheat embryos (Taneja and Sachar, 1976) or even a promoting effect of Actinomycin 'D' in potato roots (Birecka and Miller, 1974) on peroxidase activity or so-called peroxidase synthesis. Similarly the results of protein synthesis inhibitors are also conflicting. Cycloheximide, a potent inhibitor of protein synthesis, promoted the peroxidase synthesis in terms of increased activity in maize (Sharma et al. 1976). In cultured peanut cells, cycloheximide completely blocked the synthesis (activity) of peroxidase (van Huystee and Turcon, 1973), but did not affect its release into the medium.

Density labelling with deuterium oxide has also been employed to study the biosynthesis of peroxidase isoenzymes (Siegel and Galston, 1966; Anstine et al., 1970). By using density labelling in combination with gel electrophoresis and isopycnic sedimentation equilibrium, Anstine et al. (1970) demonstrated the synthesis of new peroxidase isoenzymes by germinating barley embryos.

The only report of the in vitro synthesis of peroxidase is derived from the work by Stephan and van Huystee (1980, 1981). By isolating free and membrane bound polysomes, they showed that the membrane bound polysomes could synthesize twice as much cationic peroxidase as the free polysomes.

1.6 Subcellular localization and biosynthetic pathway in plant cells

The subcellular localization of peroxidase, has been undertaken with the help of electron donor compounds which develop coloured products visible in sections. This simple means of detection has led to many studies with conflicting results (Table 1). As summarized in Table 1, peroxidase could be detected in nearly all intracellular organelles and compartments. This has also led to the same questions raised earlier regarding the number of isoenzymes. Is it possible that such a reactive enzyme is so widely distributed (Gaspar et al., 1982)? What then is the possible significance of its presence? The answer to these questions is not easy to obtain and will be discussed in the following paragraphs.

The only unanimity is based on the occurrence of peroxidase which seems to be in its association/presence in the ribosomes and endoplasmic reticulum (Pennon et al., 1970; van Huystee, 1978; Zaar, 1979). Pennon et al. (1970) reported the association of three peroxidase isoenzymes (one anionic and two cationic) with the ribosomes of lentil roots. Based on incorporation of ^{14}C-glycine they reported that the cationic peroxidases turned over more rapidly than the anionic one. They also suggested that IAA induced the synthesis of cationic peroxidases and not the anionic peroxidase. Zaar (1979) working with root hairs of cress

Table 1. A representative survey of literature for the distribution of peroxidase in the cellular compartments/organelles.

Workers	Technique Used	Cell organelles/compartments						
		cytoplasm	cell wall/ plasma-lemma	plastids	nuclei/ chromo-somes	mito-chondria	peroxi-somes	Endoplasmic reticulum/ ribosomes
Parish (1972)	Density Gradient Centrifugation & Enzyme assays	+	*	-	*	*	-	*
Darimont and Baxter (1973)	Density Gradient Centrifugation & Electron Micro-scopy	*	*	*	*	+	*	+
Raa (1973)	Electron Microscopy	+	+	*	+	*	*	*
Goff (1975)	Light and Electron Microscopy	+	+	*	-	+	*	+
Pennel and Greppin (1979)	Density Gradient Centrifugation & Enzyme Assay	+	+	-	-	+	*	+
Webster <u>et al</u>. (1976)	Electron Microscopy	+	+	-	-	+	*	+
Catesson (1980)	Light microscopy	*	+	*	*	*	*	*

+ - peroxidase activity associated with the organelle — - - peroxidase activity not detected in the organelle
* - no mention is made in the paper

(*Lepidium sativum* L.) found by assays with 3,3'-diaminobenzidine that peroxidase is associated with ribosomes. In fact he has proposed a biosynthetic pathway for peroxidase (?) monitoring $^{55}Fe^{++}$ protoheme content, in the cell walls, after feeding the root hairs with labelled $^{55}Fe^{++}$. The procedure is not specific for peroxidase and may represent data for all hemoproteins of which peroxidase may be one. Even in the case of the chloroplast membrane many hemoproteins have been shown to occur (Hoyer-Hansen, 1980).

The results from literature presented above, show that the major problem has been the identification of peroxidase as a single protein. In most of the studies the identification is based on the peroxidase activity (e.g. development of colour by electron donors such as guaiacol, benzidine based compounds, 'o' dianisidine etc.). The enzymatic activity is influenced by various factors such as pH, presence or absence of cofactors, inhibitors etc. Moreover, exogenous H_2O_2 is used for all assays and localization. In the experiments dealing with localization, other hemoproteins may also react (Hoyer-Hansen, 1980), if consideration to pH, temperature, percent aldehyde (in electron microscopic observations) is not monitored carefully.

Most of these problems associated with the localization and detection based on enzymatic activity can be overcome by using the immunochemical techniques. In immunological techniques, antibodies recognize the specific antigen against which they were raised. Antibodies have been raised against the cationic peroxidase from peanut cells in culture as described below, and used for studies on peroxidase, in this laboratory (van Huystee and Cairns, 1982).

1.7 Peanut cell suspension culture - The system of investigation

Peroxidase is an enzyme and therefore usually occurs in very low concentrations. Braithwaite (1976) could extract 200 mg of cationic peroxidase from 50 Kg of horseradish roots, the richest source of peroxidase. Peanut cells in culture selectively secrete peroxidase into the medium that supports their growth (van Huystee and Turcon, 1973). A cationic fraction isolated from this medium, accounts for three quarters of the peroxidase activity in the medium (Maldonado and van Huystee, 1980). This cationic fraction was purified to apparent homogeneity from the medium of cultured cells in a relatively few steps (Maldonado and van Huystee, 1980). Cationic peroxidase is also the most abundant isoenzyme in HRP (Selvendran and O'Neill, 1980). Moreover, Pennon _et al._ (1970) reported that IAA induced the _de novo_ synthesis of cationic peroxidases and not anionic. Hence, it is considered tentatively that cationic peroxidases may have a greater role to play in growth and differentiation in plants through IAA. Antibodies have been raised against this cationic peroxidase (van Huystee and Maldonado, 1982). Using these antibodies both _in vivo_ and _in vitro_, it was shown that this protein molecule accounted for two percent of total protein synthesis in peanut cells in culture (Stephan and van Huystee, 1980, 1981; van Huystee and Lobarzewski, 1982). Thus, peanut cells in culture provide a suitable system for studies on peroxidase biosynthesis and regulation. Moreover, cell suspension cultures have some definitive advantages over working with whole plant systems (Ludden and Carlson, 1980). Plant cells can be grown in a well defined sterile medium on a large scale. Large population of plant cells can therefore, be obtained in a

relatively homogenous state, as compared to whole plant (Vasil, 1980; Steward, 1983).

1.8 Research goals

Using the peanut cell suspension culture, the following aspects of peroxidase were intended to be explored in this presentation, using immunological techniques.

(i) The intracellular localization of peroxidase in peanut cells in culture, in order to elucidate the biosynthetic pathway of cationic peroxidase, using cationic peroxidase specific antibodies.

(ii) The nature and biogenesis of the heme moiety in peroxidase, with special reference to the precursors for the heme moiety.

(iii) To project on the functions of peroxidase in plant cells, based on the results obtained in this investigation.

CHAPTER 2

MATERIAL AND METHODS

2.1 Culturing of cells

Peanut calli and cells were derived from cotyledon slices of Virginia 56R type peanuts (*Arachis hypogeae* L.) as described by Verma and van Huystee (1970). The cells were maintained routinely over a 14 day culture period in Linsmaier and Skoog (1965) medium on a gyratory shaker as described by Kossatz and van Huystee (1976).

2.2 Source of peroxidase

Spent medium obtained after 14 days of culture was filtered over a Whatman No. 1 filter paper and the filtered medium was stored at 4°C till further use.

2.3 Purification of cationic peroxidase

The cationic fraction of peanut peroxidase was purified according to the method of Maldonado and van Huystee (1980) with slight modifications (Fig. 2). The supernatant obtained after centrifugation of dialysate at pH 5.0 (Fig. 2) was loaded onto a carboxy methyl cellulose (CMC) column for ion exchange chromatography.

2.3.1 Ion-exchange chromatography

Carboxy methyl cellulose was regenerated/precycled according to the manufacturer's instructions. A column, 2x15 cm, was packed with 50 to

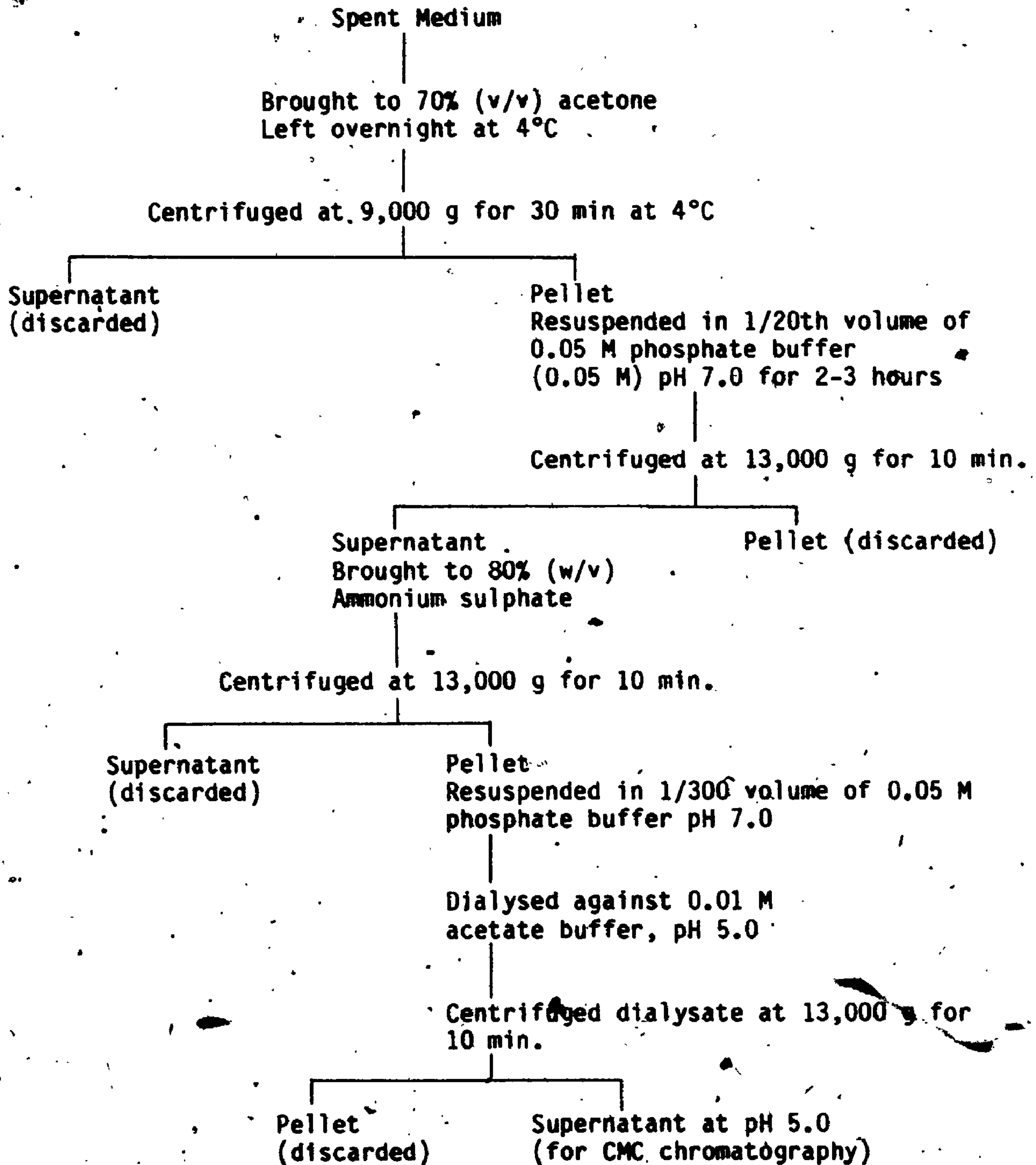


Figure 2. Flow sheet of extraction and preliminary purification of cationic peroxidase from peanut cells in culture.

60 ml of regenerated CMC at a flow rate of 1.5 to 2 $ml.min^{-1}$ at room temperature. The column was washed with 100-200 ml of 0.01 M acetate buffer pH 5.0 at a flow rate of 1 ml. min^{-1}. The sample (Fig. 2) was briefly brought to room temperature and loaded onto the column. Anionic proteins were removed by washing the CMC with 150 ml of 0.01 M acetate buffer, until the A_{280nm} of the effluent returned to the baseline. The anionic proteins were stored at 4°C for the isolation of the anionic fraction of peroxidase as discussed in Chapter 7. A gradient of 0.01 M to 0.25 M acetate buffer pH 5.0 (250 ml each) was applied to elute the cationic fraction. Fractions were collected at 5 minute intervals. The effluent of the column was monitored at A_{280nm} with an ISCO model UA-2 Ultraviolet analyser linked to a chart recorder. Subsequently the A_{407nm} and A_{280nm} for individual fractions collected from the area that had shown A_{280nm} on the chart, was measured with a Pye-Unicam spectrophotometer to calculate the Reinheitzahl (RZ) values. The absorption at 407 nm reflected the concentration of heme moiety.

Fractions with RZ value of more than 1.5 and less than 3.0 were pooled and precipitated with 80% (w/v) ammonium sulphate for 1 hour. The pellet obtained after centrifugation at 13,000 g for 10 minutes was dissolved in 0.01 M acetate buffer, pH 5.0 and dialysed overnight, against 5 litres 0.01 M acetate buffer, pH 5.0 to remove ammonium sulphate. The dialysate was centrifuged at 13,000 g for 10 minutes and the pellet, if any, was discarded. The supernatant was again chromatographed over another CMC column. The cationic peroxidase from this second column was eluted with a gradient of 0.01 to 0.08 M acetate buffer, pH 5.0, (250 ml each). The conditions of chromatography and purity measurements in terms of RZ value of peroxidase were as described above.

This procedure generally yielded peroxidase of high purity (RZ > 3). A carboxy methyl sephadex (CMS) column was used later in this study, since the flow rates were greater than that of CMC. The separation process was quick and purity was comparable to CMC, but the samples were five to ten times more dilute than obtained from CMC column. It was suggested to use CMS as the first column and CMC from Biorad may be used for purification in the second column.

2.4 Assay of peroxidase enzyme activity

2.4.1 α-aminoantipyrine as substrate

The method was adapted from Worthington Enzyme Manual (1978). An appropriate dilution of peroxidase in 0.1 ml was incubated with 0.0025 M phenol-aminoantipyrine solution (1.4 ml) with 0.0017 M H_2O_2 (1.5 ml) in a cuvette in a recording spectrophotometer. The reference cuvette had phenol-aminoantipyrine and H_2O_2 but no peroxidase. The change in absorbance at 510 nm for first 4 minutes was recorded. The specific peroxidase activity was calculated as below:

$$\text{E.U. mg}^{-1}\text{ protein} = \frac{\Delta A_{510nm}\ \text{min}^{-1}}{6.58 \times \text{mg enzyme, ml}^{-1}\text{ reaction mixture}}$$

2.4.2 Guaiacol as substrate

The method of Racusen and Foote (1965) was followed. An appropriate dilution of peroxidase in 0.1 ml of 0.05 M phosphate buffer, pH 7.0 was allowed to react with 15 mM Guaiacol and H_2O_2, respectively,

in a total volume of 3 ml in a cuvette of a recording spectrophotometer. The change in absorbance at 460 nm against a reference cuvette containing essentially the same reactants except the peroxidase, was recorded over a period of 5 minutes. The change in absorbance at 460 nm per minute was calculated from the linear portion of the curve. One enzyme unit (E.U.) was defined as a change of one absorbance unit at 460 nm per minute.

2.4.3 Eugenol as substrate

The method of Srivastava and van Huystee (1977a) was followed. To an appropriate dilution of peroxidase in 0.1 ml of 0.05 M phosphate buffer, pH 7, was added 1.6 mM eugenol and 15 mM H_2O_2 in a total volume of 3 ml. The change in absorbance at 425 nm, in a cuvette containing eugenol and H_2O_2, was followed for 5 minutes in a recording spectrophotometer. The change in absorbance at 425 nm per minute was calculated as in section 2.4.2. One E.U. was defined as change in one absorbance unit per minute at 425 nm.

2.5 Assay of IAA-oxidase activity

Indole-3-acetic acid was used as the substrate for the assay of IAA-oxidase activity. After the enzyme reaction, the remaining IAA was determined by a colorimetric method of Gordon and Weber (1951). To three ml of citrate-phosphate buffer (Sober, 1968) at the appropriate pH, was added the requisite dilution of peroxidase in 0.5 ml and allowed to incubate with the buffer for 5 minutes at room temperature. This mixture was reacted with 100 μM IAA (final concentration) in 0.2 ml at 25 to 30°C for 15 to 30 minutes. The reaction was stopped by adding 4

ml of Salkowski reagent (Gordon and Weber, 1951). This was incubated in the dark for 30 minutes. The absorbance of the pink colour that developed due to residual IAA, was measured at 530 nm. The amount of IAA in the mixture was calculated from a standard curve.

Specific IAA-oxidase activity was calculated as the amount of IAA oxidized, mg^{-1} protein, min^{-1}.

2.6 Assay of protein

Protein assays were performed according to the method of Lowry *et al*. (1951).

2.7 S.D.S. Polyacrylamide gel electrophoresis (SDS-PAGE)

2.7.1 Gel preparation

Dissociating PAGE in the presence of sodium-lauryl-sulfate (SDS) was performed, essentially according to the method of Laemmli (1970). Slab gels (14 cm x 14 cm x 1.5 mm) were used. The ratio of acrylamide to methylene bisacrylamide in total acrylamide was kept constant at 37:1 by weight for the separation and stacking gels.

An 8.75% separation gel contained 0.375 M Tris-HCl buffer, pH 8.8; 0.1% w/v SDS, 10% w/v total acrylamide, 0.1% v/v TEMED and 0.1% w/v ammonium persulphate. TEMED and Ammonium per sulphate were added prior to pouring the solution into the mold for chemical polymerization. The gel was overlayed with distilled water.

To prepare a 7.5 to 15% gradient gel which was used and found satisfactory for peroxidase separation the appropriate acrylamide concentrations were prepared as described in Table 2. Fifteen ml of

Table 2. Gel mixtures for gradient gels (15-7.5% acrylamide).

Component	Acrylamide concentration	
	Dense (15%)	Light (7.5%)
30.8% Acrylamide-bisacrylamide	7.5 ml	3.75 ml
1.875 M Tris-Cl (pH 8.8) and 0.5% SDS	3.0 ml	3.0 ml
60% Sucrose	4.42 ml	1.25 ml
Water	-	6.92 ml
TEMED	5.0 μl	8.0 μl
Ammonium per sulphate	75.0 μl	75.0 μl
TOTAL VOLUME	15 ml	15 ml

each solution was placed in a gradient mixer with the dense solution in the mixing arm. To prevent polymerization in the gradient maker, it was kept on ice. Sucrose was added to stabilize the gradient. The gradient was pumped into the mold at a speed of 2 ml, min^{-1}. After the gel was poured, the gel mixture was overlayed with distilled water. It was allowed to polymerize for 40 to 45 minutes at room temperature.

After polymerization, the water was removed by a hypodermal syringe attached to a thin intravenous tubing. The stacking gel containing 5% total acrylamide, 0.1% w/v SDS, 0.125 M Tris-HCl, pH 6.8, 0.1% v/v TEMED and 0.1% (w/v) ammonium persulphate was overlayed on the separating gel. A comb with 10 slots was then positioned in the stacking gel. This was allowed to polymerize for 30 to 35 minutes.

2.7.2 Sample preparation

An aliquot containing the proteins was precipitated with 2 volumes of methanol over ice for 1 hour. The pellet was obtained by centrifuging at 7,000 g for 2 minutes. The pellet was dissolved in sample buffer (0.05 M Tris-Cl, pH 6.8; 1% w/v SDS; 1% v/v, β mercaptoethanol; 10% v/v glycerol and 0.0025% w/v bromophenol blue) and heated on a boiling water bath for 4 to 5 minutes. According to the requirements of the experiment, 10 to 15 μg pure protein preparations and 100-150 μg crude protein preparation in no more than 50 μl per channel were loaded on to the gel.

The running buffer was 0.025 M Tris pH 8.3; 0.192 M glycine, 0.1% (w/v) SDS and 2 mM EDTA. A current of 15 mA per slab was applied for stacking

(30 minutes) and then switched to 24 mA per slab for 5 to 6 hours for electrophoresis.

2.7.3 Staining of gels

2.7.3.1 Protein-stain

Upon completion of electrophoresis, the slab gel was submerged in 25% isopropanol-10% acetic acid for 2 to 3 hours to fix the proteins and remove the SDS. The gels were stained for 2 hours with 0.1% Coomassie brilliant blue-R in 40% methanol, and 10% acetic acid (v/v). The gel was destained with 20% methanol and 7.5% acetic acid (v/v).

2.7.3.2 Hemoprotein stain

The method of Hoyer-Hansen (1980) using TMBZ as the staining reagent, was followed. Immediately after electrophoresis was completed, gels were immersed in a solution comprising of 3 parts 6.3 mM TMBZ in methanol and 7 parts 0.25 M acetate buffer pH 5.0. The gel in this solution was allowed to develop in the dark at room temperature with gentle shaking for 2 hours. Hydrogen peroxide was added to a final concentration of 3 mM . A blue colour was visible after 5 minutes and intensity of the colour increased slightly up to an hour. The gels were washed with a solution comprising of 3 parts of isopropanol and 7 parts of 0.25 M acetate buffer pH 5.0 to remove the excess TMBZ. The washing procedure was repeated once or twice, after which the gels were photographed. After marking the place for hemoprotein bands the same gels were stained for proteins with Coomassie blue as described above (section 2.7.3.1).

2.8 Immunological techniques

2.8.1 Antiserum preparation

At least one New Zealand rabbit was subcutaneously injected in the thigh with an emulsion of 50% v/v complete Freunds adjuvant containing 1.5 mg of purified cationic peroxidase (RZ < 3) in 0.05 M phosphate buffer, pH 7.0. The injection was repeated after two weeks to give a booster dose. Two weeks after the second injection, 30 to 40 ml of blood was collected by bleeding from the vein on the external side of the ear. The blood was allowed to clot for 2 hours at room temperature and then overnight at 4°C. Next day the serum was separated from the clotted red blood cells by centrifugation at 15,000 g for 15 minutes. The supernatant was the serum, which contained the antibodies. It was stored in small aliquots (2.5-3 ml) at -70°C.

2.8.2 Ouchterlony immunodiffusion assay

Twenty ml of 1.5% w/v of special Agar-Noble (Difco) was prepared in veronal buffer (0.05 M sodium barbiturate buffer, pH 8.2) by heating to 90°C. The agar solution at 60°C, was poured over a glass plate (8 x 9 cm). When the gel had formed, wells were cut and the samples as well as the antiserum were placed according to the requirements of the experiment. The gels on glass plate along with the samples in the wells were kept in a humidified petri dish (9 cm diameter). The antiserum and antigens were allowed to diffuse and peact overnight.

2.8.3 One dimensional immunoelectrophoresis

For immunoelectrophoresis, the samples were placed in the wells in the center of the plate and electrophoresed in 60 mA per gel for four

hours at room temperature. Gels were connected to the veronal buffer in the reservoir by means of paper wicks. Each wick consisted of six thickness of Whatman number 1, filter paper. At the end of the electrophoresis troughs in the agar were cut out, parallel to the antigen migration (Fig. 3) and filled with the antiserum or antigen as needed for the experiment. The antigen and antiserum were allowed to diffuse for overnight (14 to 16 hours).

2.8.4 Washing and drying the gels

After immunodiffusion, the gels were extensively washed with repeated changes of 0.015 M sodium chloride for 3 to 4 days to remove all proteins, not forming the immunoprecipitin reaction arc. After washing the gels were wrapped in a Whatman number 1 filter paper and dried at room temperature. After drying the gel was stained.

2.8.5 Staining the gel

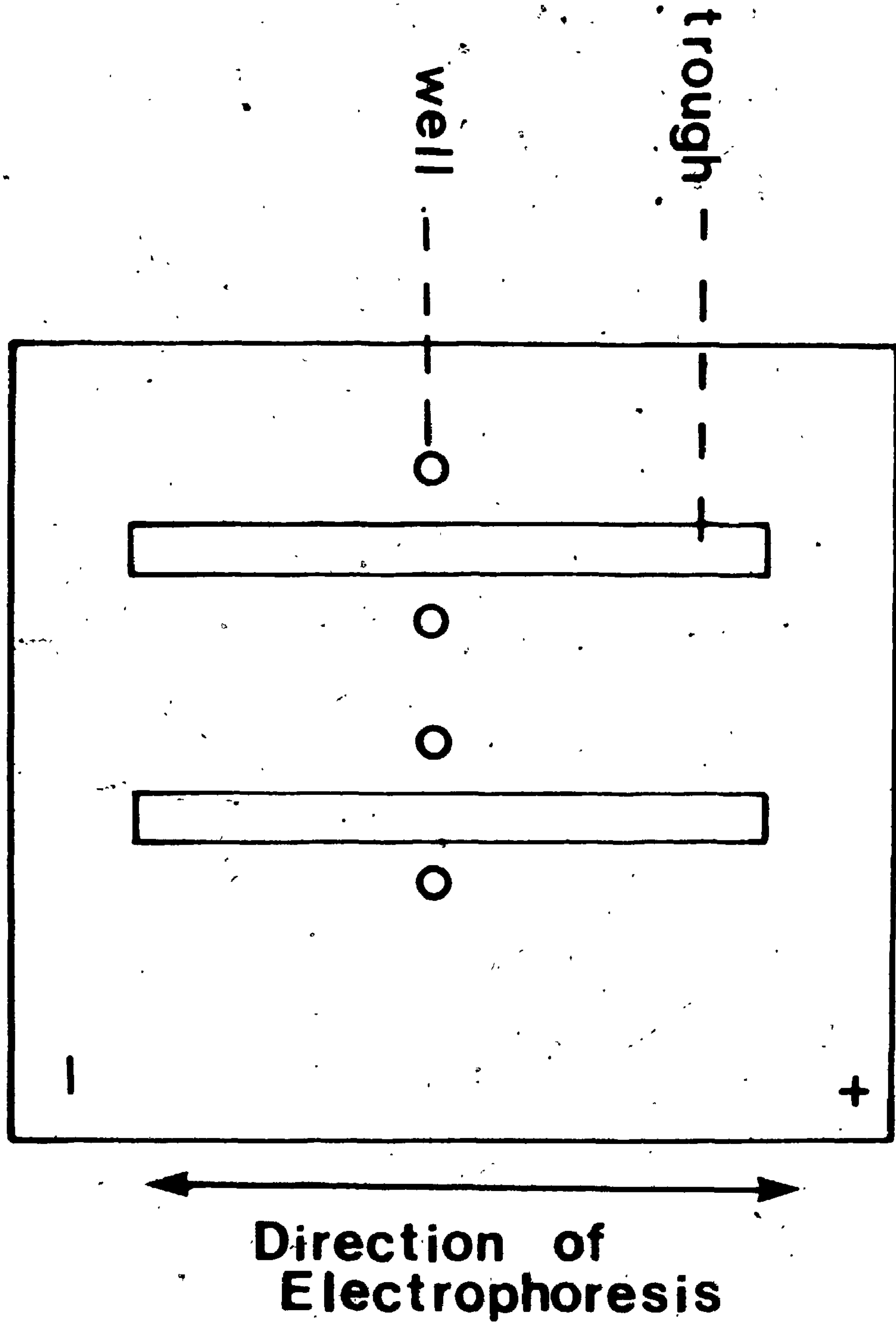
2.8.5.1 Protein

Protein staining was performed as described for SDS-PAGE in section 2.7.3.1. However, the time for staining was only 10 to 15 minutes. The destaining procedure was exactly as described before.

2.8.5.2 Peroxidase stain

Schrauwen's (1966) method was used for the staining of peroxidase on dried gels. The staining solution comprised of 20 ml of 0.5 M acetate buffer, pH 5, 30 ml distilled water, 2.5 ml of 0.005 M manganese sulphate, 2.5 ml of 0.3% H_2O_2 and 6.25 ml of benzidine-guaiacol (0.4%

Figure 3. Schematic representation of the gel for one dimensional immunoelectrophoresis. Sample to be electrophoresed was placed in the well. After the electrophoretic migration had taken place, the antiserum or IgG were placed in the trough and the process of immunodiffusion was allowed to occur overnight.



well
trough
+
Direction of Electrophoresis

benzidine and 0.25% guaiacol prepared together in 10% acetic acid). After 5 to 10 minutes of staining, the peroxidase precipitin arcs appeared brown. The gels were destained in 7.5% acetic acid to remove excess stain.

After staining the gels on plates could be dried and stored at room temperature.

2.9 Ratio of peroxidase versus protein synthesis

The cells in suspension culture were usually incubated with 1 M Bq of ^{35}S methionine for 2 hours to allow synthesis of labelled peroxidase and proteins. In the case of leaves, the petioles of three or four compound leaves were dipped in 2 to 3 ml of Linsmaier and Skoog (1965) medium containing 2.25 M Bq of ^{35}S methionine for 12 to 14 hours. After incubation the cells were filtered under vacuum and washed with distilled water to remove any adsorbed peroxidase. However, before washing the cells, the medium was removed in order to carry out radioimmunoassays on the amount of peroxidase released.

2.9.1 Extraction of proteins and peroxidase

The tissue, cells or callus were extracted in 0.05 M phosphate buffer, pH 7.0, containing 0.005 M sodium metabisulphite (ratio of tissue to buffer 1:3, weight/volume) and homogenized in a prechilled pestle and mortar with sand. The homogenate was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was saved as the low ionic extract. The pellet was re-extracted with the same buffer and the second supernatant was pooled with the low ionic extract. The pellet

remaining from low ionic extraction was then extracted with the same buffer, supplemented with 0.8 M potassium chloride. The pellet after dispersal in the high salt buffer was kept on ice at 4°C for 30 minutes. The slurry was centrifuged at 12,000 g for 10 minutes at 4°C. This supernatant served as the high ionic extract.

2.9.2 Estimation of protein synthesis

The filter paper disc method of Mans and Novelli (1961) was followed and essentially consisted of the following steps. An aliquot of 25 to 100 μl of extract was loaded on a Schleicher and Schuell filter paper disc (dia. 12.7 mm). After evaporating excess liquid under warm air the filter papers with extracts, were immersed in an ice cold solution of 10% TCA and 0.1 M of the amino acid used for labelling (ca 3 ml solution, $disc^{-1}$). All the discs were put in one beaker and left in the TCA solution for one hour, on ice. Next, the TCA solution was replaced by 5% TCA for 15 minutes. After draining this TCA, the discs were incubated in 5% TCA at 90°C for 30 minutes. The discs were again washed with 5% TCA. These TCA washed discs were suspended in ether-ethanol mixture (volume/volume) and incubated at 37°C for 30 minutes. Finally the discs were resuspended in ethyl ether for 15 minutes at room temperature. Frequently another wash with ethyl ether was given and the discs were dried under low heat. The dried discs were transferred to a scintillation vial and 5 ml of aquasol was added and the radioactivity of the sample measured.

2.9.3 Estimation of peroxidase synthesis

An aliquot of 0.5 ml of radioactive cell or tissue extract or medium was added to 200 μl of antiserum, prepared as in section 2.8.1. To this mixture was added 50 μl of 0.01 M phosphate buffer (pH 7.5) containing 0.15 M sodium chloride, 10% Triton X-100 and 10% deoxycholic acid (DOC) and 30 μl (0.15 μg protein) of pure cationic peroxidase and incubated for 2 hours at room temperature and 12 to 14 hours (overnight) at 6 to 10°C. The immunoprecipitate so obtained was raised on a cushion of 400 μl of 1 M sucrose containing 1% Triton-X 100 and 1% DOC introduced by a hypodermal syringe. This was centrifuged at 7,000 g for 7 minutes and the supernatant was discarded. The pellet was washed twice by suspending in a solution of 0.85% sodium chloride, 1% Triton X-100 and 1% DOC and centrifuging at 7,000 g for 3 minutes. The washed pellet was solubilized in 5 ml aquasol in a scintillation vial and the radioactivity was measured.

The amount of peroxidase synthesis was expressed as cpms in immunoprecipitate, mg^{-1} protein, or f moles of ^{35}S methionine incorporated in peroxidase, mg^{-1} protein.

Ratio of peroxidase to total protein synthesis is:

$$= \frac{\text{cpm in immunoprecipitate}}{\text{cpm in TCA-precipitate}}$$

Peroxidase in the experimental part of this thesis refers to cationic fraction of peroxidase, unless mentioned otherwise.

CHAPTER 3

THE SYSTEM OF INVESTIGATION

3.1 Introduction

A peanut cell suspension culture was used as a biological system for studies on peroxidase described in this research. All cell culture studies assume, that the basic conditions in culture are essentially similar to that of cells in intact plant. However, doubts still persist as to the generation of mutations in gene products during prolonged culture period (Meins Jr., 1983; Chaleff, 1983; Chaleff and Ray, 1984). Intraspecies (Shannon et al., 1966; Stephan and van Huystee, 1981) as well as interspecies; (Welinder and Mazza, 1975; Lobarzowski and van Huystee, 1982; van Huystee and Maldonado, 1982) variations have been observed in relation to peroxidase structure. Therefore, in this chapter, the similarities of peanut cells in suspension culture to the intact peanut plant shall be stressed, specifically as to the facets (peroxidase secretion and the immunological relatedness of peroxidase from plants and that of their cultured cells) intended to be studied. In addition, the advantages offered by the peanut cells in suspension cultures for studies on peroxidase will be discussed.

3.2 Materials and methods

3.2.1 Vacuum infiltration for extraction of intercellular peroxidase

The technique of Rathmell and Sequeira (1974) with slight

modifications was used. The leaves of 2 to 3 months old peanut (Virginia 56R) plants were vacuum infiltrated with 0.05 M phosphate buffer (pH 7.0) three times for 5 minutes. The intercellular liquid was recovered after centrifugation of the whole leaf at 7,000 g for 20 minutes. Approximately 1 ml of intercellular fluid was recovered from 20 g fresh weight of leaves.

3.2.2 Ratio of peroxidase to total protein synthesis

The petioles of six compound leaves (with 3 leaflets) were dipped in 2.26 M Bq of ^{35}S methionine in 2 to 3 ml of Linsmaier and Skoog (1965) medium overnight as described in section 2.9.1. Ten ml of enriched cell culture (4 days old ca ≃ 2 g fresh weight of cells) was incubated with 0.75 M Bq ^{35}S methionine for 2 hours. Total proteins including peroxidase were extracted from the leaves and cells as described in section 2.9.1.

Total proteins were precipitated with 10% TCA and peroxidase by immunoprecipitation as described in sections 2.9.2 and 2.9.3, respectively.

A similar approach was used for calculation of the ratio of peroxidase to proteins in the medium after incubating the cells with labelled amino acids for 2 hours. All the experiments were representative of at least 3 experiments with similar trends.

The SDS-PAGE was carried out as described in section 2.7.

3.3 Results and Discussion

Table 3 showed that peroxidase activity could be detected in all peanut plant parts in addition to the cultured cells and callus.

Table 3. Specific peroxidase activity in the extracts of peanut plant organs, cultured calli, cells and medium that supported the growth of cells in culture.

Plant Part (Constituent)*	Per ml Extract		Specific Activity
	Protein (μg)	Peroxidase Activity (E.U.)***	
Root	600	14.6	23
Stem	348	8.0	23
Leaf	392	2.3	6
Leaf Intercellular spaces**	165	8.0	48
Callus	364	132.0	362
Cells (cultured) 14 days old	356	28.0	78
Medium**	50	45.0	900

* For extraction of peroxidase from roots, stem, leaves, calli and cells, app. 1 g was used.
Prior to extraction cells were rinsed repeatedly with water to remove any adsorbed peroxidase.

** Intercellular fluid (1 ml) was obtained from app. 20 g of fresh weight of leaves. The conditioned medium and intercellular fluid were used as such for assays.

*** One enzyme unit (E.U.) of peroxidase is the change in one absorbance unit at 470 nm per minute.

However, the S.A. of peroxidase varied from part to part, leaves exhibiting the lowest value. Conversely it was shown that the S.A. in the intercellular spaces in the leaves was high, compared to that from whole leaves, thus indicating a rather selective release of peroxidase by the cells in the plant. The S.A. of the callus was higher than that of the cells in suspension culture. This may largely be due to a diffusion gradient which builds up in the callus grown stationary for at least one month versus the cells which were allowed to be continuously agitated and bathed in fresh medium every fortnight (Kossatz and van Huystee, 1976). However, the greatest S.A. was observed in the suspension medium of cultured cells. The important conclusion to be drawn from this wasthat peroxidase was secreted by cells in culture as well as in intact peanut plant. This secretion of peroxidase was not unique to peanut plants or cells in culture. Secretion of peroxidase by cells in the plant has been reported for tobacco (Rathmell and Sequeira, 1974; Bredemeijer and Blaas, 1983) and petunia (Berg and van Huystee, 1984). Regarding cultured cells, there are also various reports on peroxidase secretion such as for spinach (Fry, 1979), beet root (Gaspar et al., 1983) and carrots (Chibbar et al., 1984). In the secretory process peroxidase accumulated in the medium of cultured peanut cells. The increase in the magnitude of secretion in the suspension cultured cells might be due to the enhanced extracellular space in the medium.

3.3.1 The percentage of cationic peroxidase to total proteins in the culture medium

In Table 4, it was shown that the cationic peroxidase could constitute up to one sixth of the total proteins in the culture medium.

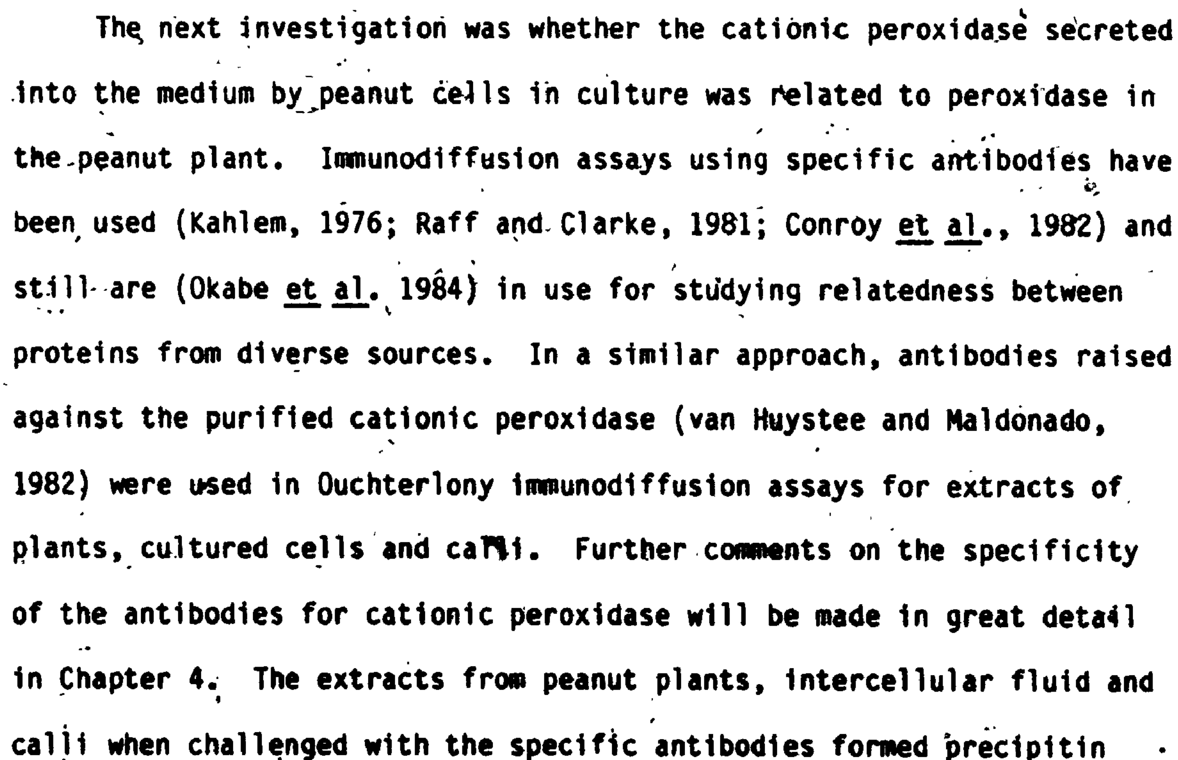
Table 4. Comparison of peroxidase to total proteins in the meidum of peanut cells in suspension culture.

Serial No.	Labelled amino acid	Volume of Medium	10^3 cpm for Total Proteins	10^3 cpm for Peroxidase	Percentage of peroxidase to Total Proteins
1	^{35}S methionine (1 M Bq)	8.0 ml	235	39.4	16
2	^{14}C leucine (0.2 M Bq)	6.0 ml	29.8	4.6	15

Ten ml of enriched cell suspension culture (4 day old ca 2 gm fresh weight of cells) were incubated with the labelled amino acids for 2 hours in light. The medium was separated from the cells by filtration. Four aliquots (2 ml for total proteins and 0.5 ml for peroxidase) were precipitated for total proteins with 10% TCA and peroxidase by immunoprecipitation. (A representative of three experiments with identical trends.)

$$\text{Percentage of peroxidase in medium} = \frac{\text{cpm in immunoprecipitate}}{\text{cpm in TCA precipitate}} \times 100$$

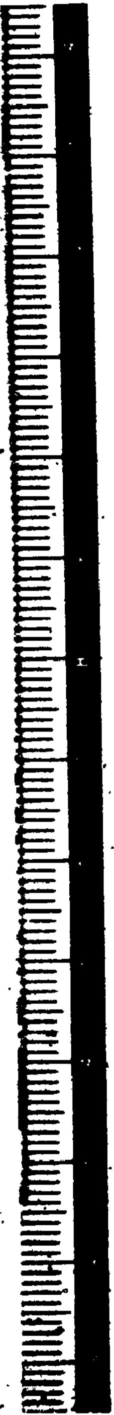
In petunia, based on peroxidase enzyme activity, it has been reported that total peroxidase forms 15 percent of total proteins in the intercellular spaces of stem (Berg and van Huystee, 1984). The secretion of peroxidase by peanut cells is rather selective and an energy dependent process (van Huystee and Lobarzewski, 1982). The medium of cultured peanut cells contained few proteins as seen from SDS-PAGE (Fig. 4). This was in good agreement with earlier studies (Stephan and van Huystee, 1980). In addition the cationic peroxidase purified from the spent medium was a relatively homogenous protein, as only a single protein band could be seen on SDS-PAGE (Fig. 2). No additional bands were visible with 35 μg proteins in each channel.



3.3.2 <u>Homology of cationic peroxidase from cultured cells to that from peanut plant</u>

The next investigation was whether the cationic peroxidase secreted into the medium by peanut cells in culture was related to peroxidase in the peanut plant. Immunodiffusion assays using specific antibodies have been used (Kahlem, 1976; Raff and Clarke, 1981; Conroy et al., 1982) and still are (Okabe et al. 1984) in use for studying relatedness between proteins from diverse sources. In a similar approach, antibodies raised against the purified cationic peroxidase (van Huystee and Maldonado, 1982) were used in Ouchterlony immunodiffusion assays for extracts of plants, cultured cells and calli. Further comments on the specificity of the antibodies for cationic peroxidase will be made in great detail in Chapter 4. The extracts from peanut plants, intercellular fluid and calli when challenged with the specific antibodies formed precipitin

Figure 4. SDS-PAGE on a 7.5-15% gradient gel of 35 (channel a) and 25 μg (channel b) of cationic peroxidase; 150 (channel c) and 75 μg (channel d) medium proteins. Channel e is molecular weight markers phosphorylase b (94kD); bovine serum albumin (67kD); ovalbumin (43kD); carbonic anhydrase (30kD); soybean trypsin inhibitor (20kD) and α-lactalbumin (16.4kD).



arc, with all (Fig. 5), except extracts from roots. Roots had exhibited a high S.A. of peroxidase (Table 3). The fused precipitin arcs in Fig. 5, indicate the homology of the cationic peroxidase from the cells in suspension culture to the peroxidase occurring in the intercellular fluid of leaves, callus lysate, stems and leaves. Only in the case of callus lysate did a spur occur suggesting incomplete homology (Crowle, 1975) to the peroxidase in cell suspension cultures. These results should allay the fears induced by reports on mutations of genes for protein structure regarding peroxidase that do arise in cultured calli and cells (McCoy and Bingham, 1977; Chaleff, 1981, 1983; Raff and Clarke, 1981). This also suggested that observations with peroxidase from cultured cells and the antibodies raised against it may be used for studies on the role of peroxidase in plant development (van Huystee and Cairns, 1982). However, the significance of the minor additional precipitin arc formed with the leaf extract (b, Fig. 5) was not understood.

3.3.3 Proportion of peroxidase to total protein synthesis in peanut leaf

Table 5 showed that the radioactivity incorporated in the immunoprecipitate of peroxidase was only 0.2% of that incorporated in the total proteins, in peanut leaves. Whereas, in peanut cells in suspension culture, it was 2% of that incorporated in total proteins (Table 5). Hence, it inferred that the cells in suspension culture were synthesizing approximately ten-fold more peroxidase than the plant. In comparison to carrot cells in culture, which are used extensively for *in*

Figure 5. Ouchterlony immunodiffusion of extracts of 4 month old peanut plant parts with antibodies raised against the cationic peanut peroxidase. The center well contains the antiserum raised against the cationic peroxidase. The peripheral wells contain extracts from (a) roots 25 μg; (b) leaves 50 μg; (c) stem 50 μg; (d) intercellular fluid 5 μg; and (e) callus cultured *in vitro* - 35 μg, protein, respectively, in each well.

The gels were stained for protein with Coomassie blue and washed with 7% acetic acid.

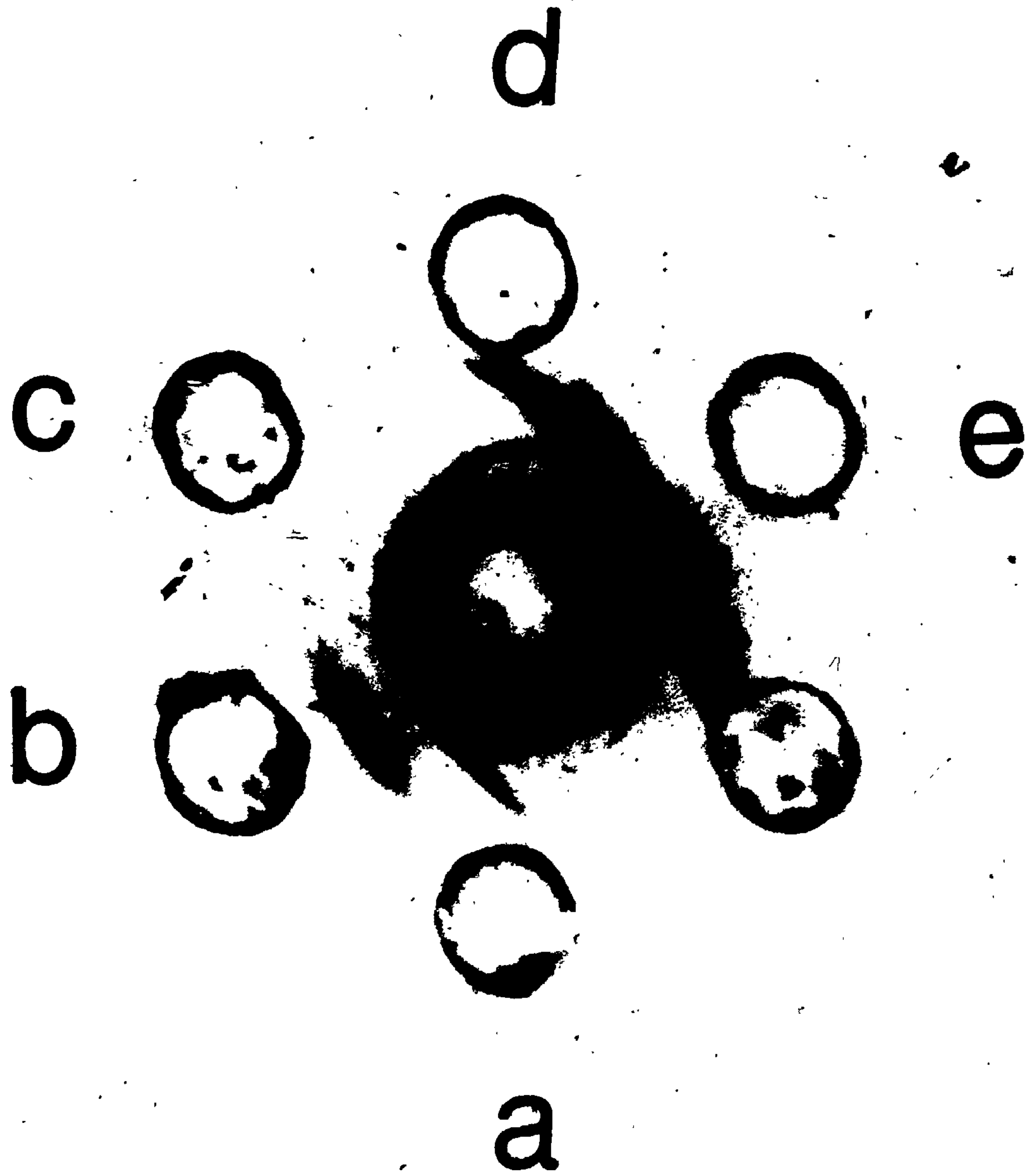

d
c
e
b
a

Table 5. The synthesis of peroxidase and proteins in peanut leaves and cells in suspension culture.

Serial No.	System	Total Proteins (TCA insoluble) cpm	Peroxidase (Immuno-precipitate) cpm	*Percentage of peroxidase to total proteins
1	Peanut cells in suspension culture	3.5×10^6	81.4×10^3	2.3
2	Peanut leaf	293×10^3	7×10^2	0.2

* Percentage of peroxidase to total protein synthesis =

$$\frac{\text{cpm in immunoprecipitate}}{\text{cpm in TCA percipitate}} \times 100$$

vitro studies (Steward, 1983), the peroxidase synthesis as compared to total protein synthesis is only 0.3 to 0.7% depending on the cell line (Chibbar *et al*., 1984). The peroxidase activity in the medium of cultured carrot cells is also 10 to 20 times lower than in the medium of peanut cells (Chibbar *et al*., 1984).

In conclusion, peanut cells in suspension culture were similar to peanut plant as far as the secretion of peroxidase into the extracellular spaces and peroxidase structure (immuno-determinants) were concerned. However, the ten-fold higher synthesis of peroxidase in peanut cells in suspension culture over that in peanut leaves and its selective release into the medium (van Huystee and Turcon, 1973) made it an ideal system for exploring the less studied aspects of peroxidase biosynthesis and regulation.

CHAPTER 4

IMMUNOAFFINITY STUDIES ON PEROXIDASE

4.1 Introduction

This chapter deals further with the characterization of the system of investigation particularly in terms of the antibodies specificity against the cationic peroxidase. By using the technique of immunoaffinity, monospecific IgGs had been isolated from the antiperoxidase serum. In addition, the potential use of these IgGs in one step immunoaffinity purification of peroxidase had been explored. However, the ultimate aim of these monospecific IgGs is to employ them in the isolation of mRNA for peroxidase (Boyer et al., 1983; Colbert et al., 1983)in future studies. Monospecific IgGs isolated by immunoaffinity chromatography are still considered a reasonable alternative to monoclonal antibody generation (Gershoni and Palade, 1983). In addition the monospecific IgGs against peroxidase were used to show that a ratio of 1:8 (w/w) of peroxidase to IgGs was needed to completely immunoprecipitate peroxidase.

4.2 Material and Methods

4.2.1 Materials

The cationic peroxidase (RZ > 3) from the spent medium was isolated and antibodies against it were raised in rabbits as described in section 2.3 and 2.8.1; respectively. Staphylococcus aureus bearing protein A (Immunoprecipitin) was obtained from Bethesda Research Laboratories,

U.S.A. It was also prepared in our laboratory using S. aureus strain Cowan 1 (ATCC 12598 and NCTC 8930) obtained from Dr. E. L. Medzon of the Department of Microbiology and Immunology, University of Western Ontario. The method of culture and isolation was essentially that is outlined by Kessler (1981).

4.2.2 Purification of monospecific IgGs

The procedure was basically that described by Hunt and Pratt (1979) for the purification of phytochrome, with minor modifications. One gram of the activated sepharose beads (CNBr - sepharose 4B, obtained from Pharmacia Chemicals) was washed extensively with 200 ml of 1 mM HCl. The washed sepharose was gently mixed with 2.5 ml of the cationic peroxidase fraction (7-8 mg protein and RZ > 3.0) in 0.1 M $NaHCO_3$ and left for 14 hours at 4°C. The coupling of the peroxidase to sepharose was considerably enhanced when the cationic peroxidase fraction (Maldonado and van Huystee, 1980) was precipitated with 70% acetone and then resolubilized in 0.1 M $NaHCO_3$ prior to binding. After 14 hours the beads were pelleted by centrifuging at 1000xg for 2 minutes and the colourless supernatant was decanted. The charged beads were incubated overnight with 0.1 M monoethanolamine (pH 9.0, 4 ml/ml sepharose) at 4°C. The sepharose immobilized peroxidase was packed in a column (12 x 75 mm) and washed extensively with 25 mM MOPS-Tris (pH 7.8) containing 5 mM EDTA (MTE buffer). Next, 3 M $MgCl_2$ (volume/volume sepharose) brought to pH 7.5 with Tris was percolated through the gel, immediately followed by about 30 to 40 ml of MTE buffer to remove adsorbed material on the gel. Sepharose immobilized peroxidase fraction was stored at 4°C in MTE, containing 0.02% (w/v) sodium azide.

Five ml of whole serum containing antiperoxidase IgGs was mixed with one gram sepharose immobilized cationic peroxidase and left overnight at 4°C. Most of the unbound constitutents of serum in solution were removed as the supernatant from the sepharose immobilized peroxidase by centrifugation at 1000xg for 2 minutes. The sepharose immobilized peroxidase and linked IgGs and some components of the serum remaining were packed into a column as above. Extensive washing with 10 mM MOPS-Tris (pH 7.8) followed until the A_{280} nm returned to the base line. Then the column was washed with 20 to 30 ml MTE buffer. Next the IgGs were eluted at pH 7.5 with 3 M $MgCl_2$ (vol/vol sepharose) followed immediately by 20 to 30 ml MTE buffer. Fractions with A_{280} nm > 0.02 were pooled and extensively dialysed against 4 liters of 0.25 mM MTE buffer at 4°C to remove $MgCl_2$. These IgGs were then concentrated by precipitation with 50% ammonium sulphate. The pellet was dissolved in 0.2 M $NaHCO_3$, if it was to be used for immunoaffinity purification of peroxidase, or otherwise in 0.05 M sodium phosphate buffer (pH 7.0) and dialysed against several volumes of phosphate buffer to remove residual ammonium sulphate. IgGs in small aliquots were stored at -70°C. The IgG concentration was determined using $E_{280}^{1\%}$ nm = 13.8 (Sober, 1968).

4.2.3 <u>Non-specific IgGs</u>

Non-specific IgGs were obtained from the affinity chromatography of non-immune serum over protein-A agarose (obtained from Sigma Chemical Co., St. Louis, Mo.), essentially according to the technique of Hjelm et al. (1972). One hundred mg of protein-A agarose was thoroughly washed with at least 50 ml of 0.1 M KH_2PO_4 (pH 7.0) over a scintered glass

funnel. After washing, the agarose was packed into an Econo column (10 x 0.5 cm; Biorad). The effluent from the column was passed through a peristaltic pump to an ISCO UV monitor, set at 280 nm. One ml of non-immune serum was passed over the protein-A-agarose column and non-IgG proteins were washed with phosphate buffer, till the A_{280} nm of the eluant returned to the base line. The IgGs were eluted with 0.1 M glycine-HCl (pH 3.0) buffer. The IgGs were precipitated with 50% ammonium sulphate and treated the same way as monospecific IgGs in Section 4.2.2.

The purity of proteins from both columns was checked on SDS-PAGE as described in Section 2.7. Their identification as IgGs and specificity was established by immunoelectrophoresis as described in section 2.8.3. Peroxidase activity was measured in the presence of 0.2% guaiacol as described in section 2.4.2.

4.2.4 Immunoaffinity purification of peroxidase

The purified anti-cationic peroxidase fraction IgGs were immobilized on activated sepharose as describe above in Section 4.2.4 and stored at 4°C. The protein preparation (medium or cellular proteins) resuspended in 0.1 M $NaHCO_3$ was shaken gently with the sepharose immobilized monospecific IgGs for 30 min. The beads were pelleted by centrifugation at 1000xg and the supernatant was decanted. The beads were packed into a (12 x 75 mm) column as described above and washed with glass distilled water, until the A_{280} nm returned to the base line (Fig. 9). Note that washing the Sepharose immobilized IgGs and attached peroxidase protein with MTE buffer as for purification

of IgGs and in other cases (Hunt and Pratt, 1979; Silvestrini et al., 1983), prematurely detached the peroxidase fraction from the IgGs. Hence, double distilled water was used to remove the adsorbed substances on the gel. The cationic fraction of peroxidase was eluted with 3 M $MgCl_2$ (pH 7.5) as above. This elution was immediately followed by a further wash with double distilled water, until the A_{280} nm returned to the base line. The $MgCl_2$ was removed by extensive ~~dialysis~~ against water before the purified peroxidase was employed.

4.3 Results and Discussion

4.3.1 Specificity of IgGs

The only proteins that were eluted from the sepharose immobilized cationic peroxidase fraction column by 3 M $MgCl_2$ or from the protein-A agarose column are IgGs as seen by an identical pair of protein bands following the SDS-PAGE (Fig. 6). Only two bands corresponding to the 50 kD (heavy chain) and 23 kD (light chain) could be seen as the proteins eluted from both columns (Fig. 6, channels b and c), which were similar to the ones reported by Hunt and Pratt (1979) for antiphytochrome IgGs. The results in Fig. 7, confirmed that both the proteins were IgGs, because they react with antirabbit IgG goat serum (trough 1, wells a and b; c.f. Fig. 3). However, the non-specific IgGs in trough 'a' did not react with the cationic peroxidase in wells 'c', as those of the monospecific IgGs do in the adjoining trough 'b'. This showed that the IgGs from non-immune serum eluted from protein-A-agarose column were non-specific (channel b in Fig. 6 and well a in Fig. 7) and those from sepharose-immobilized-peroxidase were specific (channel c in Fig. 6 and well b in Fig. 7).

Figure 6. Comparison of molecular weight of the non-specific (channel b) and monospecific antiperoxidase IgG (channel c). A 20 μg protein aliquot was employed and were electrophoresed on 7.5 to 15% SDS-PAGE and susbsequently stained with Coomassie blue. Channel a represents the markers, phosphorylase b (94 kD); bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); soybean trypsin inhibitor (20 kD) and lactalbumin (16.4 kD).

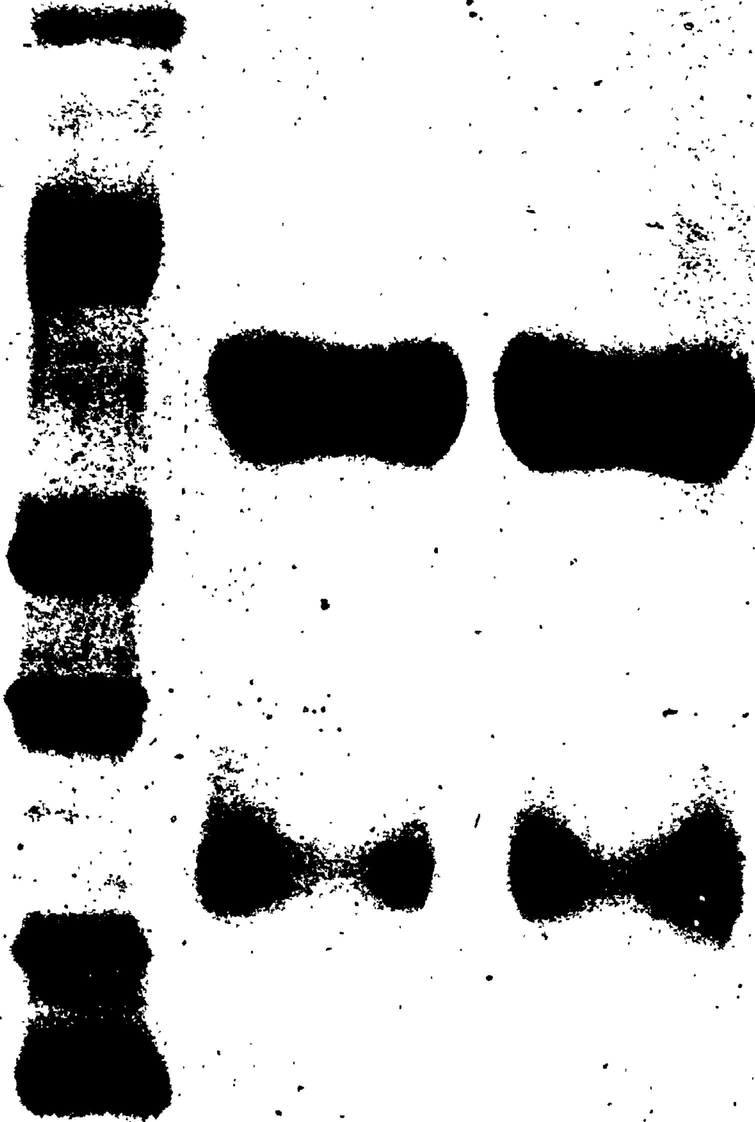

a
b
c

Figure 7. Comparisons between peroxidase specific- and non-specific rabbit IgG and their relation to antirabbit IgG goat serum. Non-specific (well a) and monospecific peroxidase (well b) IgG (10 μg each) were challenged following electrophoresis with 100 μl of antirabbit IgG goat serum (trough 1). And 10 μg cationic peanut peroxidase (well c) was challenged by either 50 μg non-specific (trough a) or peroxidase specific (trough b) IgGs.

−
1
a
b
a
b
c
c
c
+

4.3.2 Quantitative immunoprecipitation of antigens

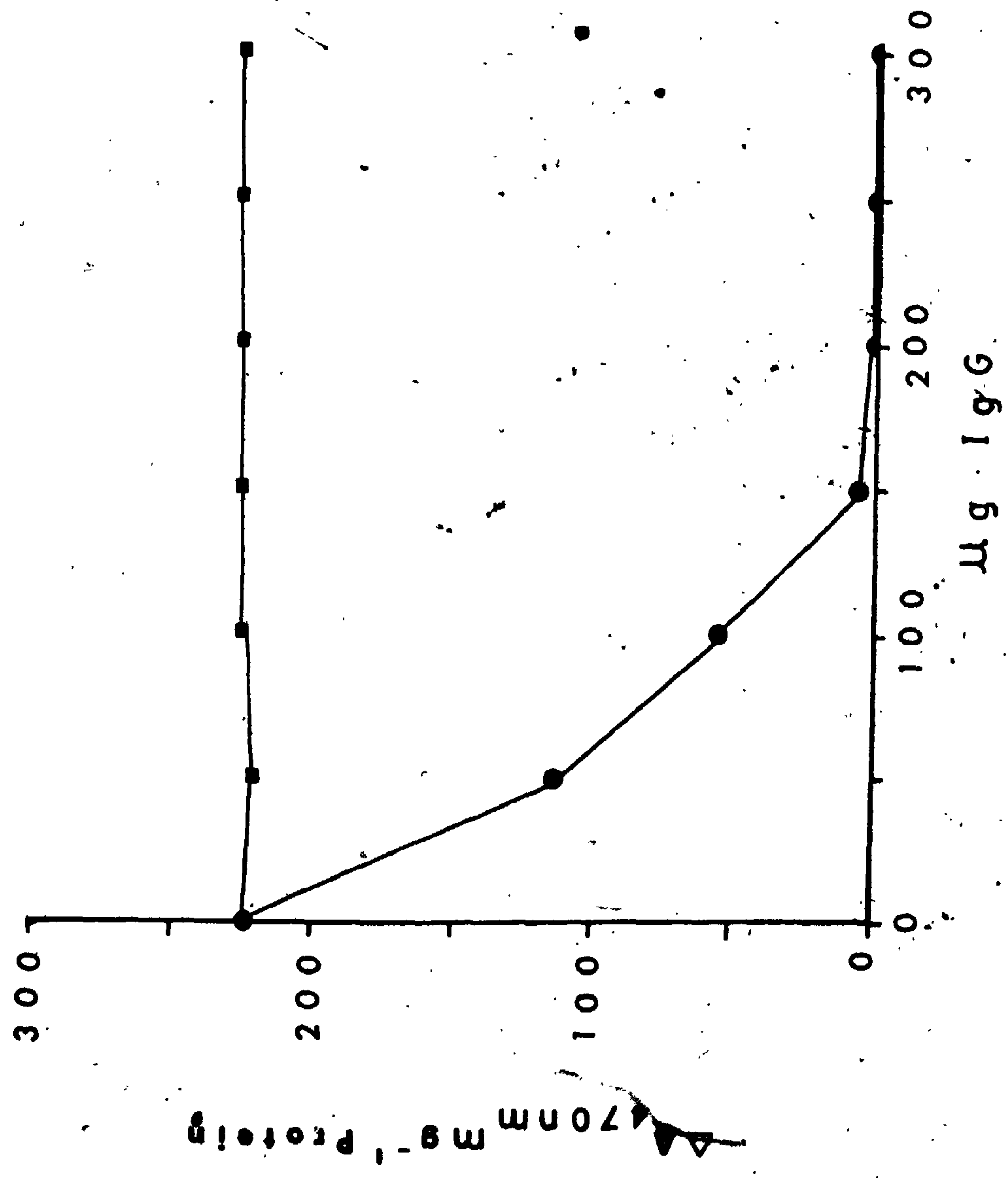
The specificity of the IgGs purified against the cationic peroxidase fraction was further examined through their capacity to pellet the soluble cationic peroxidase as antigen, in the case shown here as peroxidase activity remaining in the supernatant. The results presented in Fig. 8, showed that when a fixed amount (25 μg) of pure cationic fraction of peroxidase was titrated against varying concentrations of IgGs the peroxidase activity in the resulting supernatant decreased with increasing concentration of IgGs. At a ratio of IgG to cationic peroxidase fraction equal to 6:1 (w/w), the peroxidase fraction was totally precipitated. Hence no peroxidase activity could be detected in the supernatant (Fig. 8). Heat killed and formalin fixed Staphylococcus aureus was used for pelleting the IgG-peroxidase complex. Protein A from S. aureus has been shown to react nonspecifically with some plant glycoproteins by pelleting them along with the antigen-antibody complex (Mau and Clarke, 1983). Since peroxidase is also a glycoprotein, controls without IgGs for peroxidase were mixed with killed S. aureus cells. No decrease in peroxidase activity could be measured (Fig. 8), thus discounting the possibility of non-specific precipitation.

In conclusion, although a ratio of 6:1 was observed as a minimal concentration for the complete IgG-Ag reaction, in practice a slightly higher concentration of 8:1 of IgG to Ag was normally used.

Figure 8. Quantitative immunoprecipitation of the peroxidase by purified specific IgG.

Twenty-five μg of cationic peroxidase (RZ = 3.2) in 25 μl of 0.05 M sodium phosphate buffer (pH 7.0) was titrated against varying concentrations of purified IgG in 250 μl (●——●). After allowing incubation for 4 hr. at room temperature and 2 hr. at 4°C, 50 μl of Staphylococcus aureus was added to pellet the IgG and IgG-Ag complex. The pellet was separated by centrifugation at 15,000xg for 5 min. and peroxidase activity was measured in the supernatant.

Buffer instead of IgG added (■——■) and the same treatment as above. (A representative of 3 experiments with identical trends.)


300
200
100
0
100
200
300
μg IgG
ΔA470nm mg⁻¹ Protein

4.3.3 Immunoaffinity purification of peroxidase

Immunoaffinity techniques, using the agarose immobilized IgGs have been so far reported for purification of only a few plant proteins such as legumin (Casey, 1979), phytochrome (Hunt and Pratt, 1979) and ribulose 1,5 biphosphate carboxylase (Gray and Wildman, 1976). A method for peroxidase purification, using IgGs separated from serum proteins by CM affigel blue was reported (Lobarzewski and van Huystee, 1982). In the present report we had used the mono-specific IgGs purified as described above, for the immunoaffinity purification of peroxidase.

The protein profile of the unfractionated medium (Fig. 9B) obtained from the immunoaffinity column, showed that the first peak containing a large amount of unabsorbed proteins was eluted with water. The second peak eluted with 3 M $MgCl_2$ contained the peroxidase fraction as determined by the absorption at 407 nm and RZ value (Table 6 and its mobility on SDS-PAGE (Fig. 10, channel a). A similar elution profile was also obtained following chromatography with peanut cell extract (Fig. 9C) but the peak eluted with $MgCl_2$ containing the peroxidase fraction was small relative to the unabsorbed flow through peak. The reason for this was obvious since the peroxidase concentration in cellular extracts is low (2 percent of the total proteins) (Stephan and van Huystee, 1981) as compared to 16 percent in the medium (Chibbar and van Huystee, 1983b). Due to the low concentration of peroxidase in cell extract the protein could not readily be visualized on SDS-PAGE (Stephan and van Huystee, 1980). When the purified cationic peroxidase was passed through this column, no proteins could be detected in the area where previously the first protein peak had occurred (Fig. 9A). A large

Figure 9. Immunoaffinity of the peroxidase fraction (A); from the unfractionated medium proteins (B) and from a cultured cell extract (C).

Purified peroxidase specific IgGs (7.5 mg) were covalently linked to 0.5 g CNBr-activated sepharose as described. (9A) An aliquot of 1.5 mg of purified cationic peroxidase was mixed with the immobilized IgGs. Arrow represents the point of adding 3 M $MgCl_2$ and the peak is the cationic peroxidase fraction. (9B) A 10 mg aliquot of unfractionated medium proteins was mixed with the immobilized IgGs as above. (9C) A 20 mg protein aliquot from peanut cell extract. Note the proportion of the first flow through peak to that of the second peak eluted with $MgCl_2$.

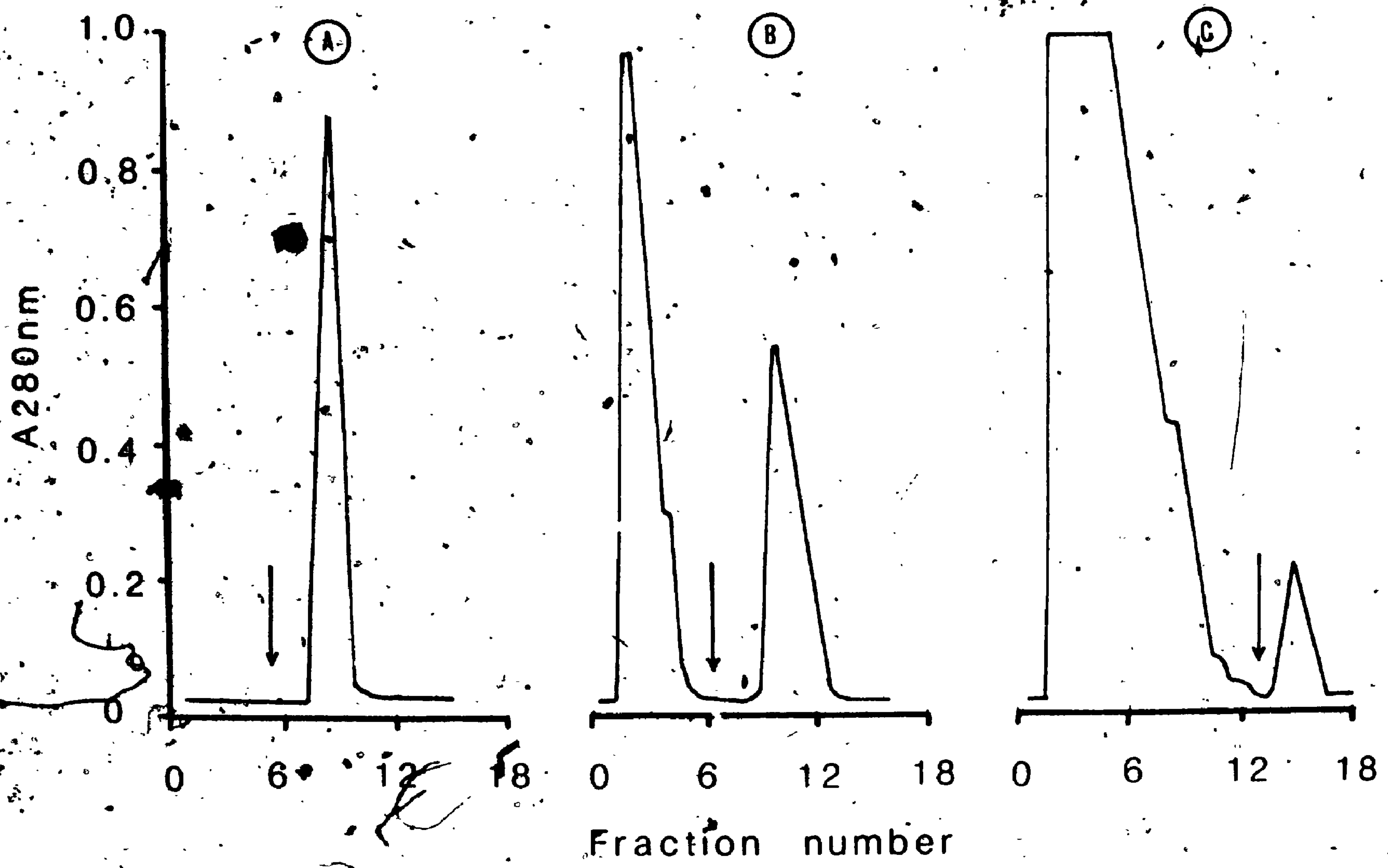

A
B
C
1.0
0.8
0.6
0.4
0.2
0
A280nm
0
6
12
18
0
6
12
18
0
6
12
18
Fraction number

Table 6. Purification of the cationic fraction of peroxidase by immunoaffinity chromatography as evaluated by RZ value.

	RZ value	
	Before*	After**
1. CMC purified cationic peroxidase	3.0	2.7[a]
2. Unfractionated me[illegible] proteins	0.6	2.0[b]
3. Cellular proteins from cultured cells	---[d]	1.8[c]

a - peak 1 of Fig. 9A.

b - peak 2 of Fig. 9B.

c - peak 2 of Fig. 9C.

d - reading at 407 nm non-detectable.

* - Before immunoaffinity chromatography.

**- After immunoaffinity chromatography.

compact protein peak could be detected when eluted with $MgCl_2$, which corresponded to the peroxidase fraction. Therefore, the proteins eluted with $MgCl_2$ appeared to be the peroxidase and the IgGs appeared to be specific for that protein.

The purity, measured in terms of RZ value (Table 6) of the cationic peroxidase purified by immunoaffinity column, did not equal that obtained by conventional CMC chromatography (Maldonado and van Huystee, 1980). In fact, the RZ value of the cationic peroxidase decreased from 3.0 to 2.7 (Table 6). However, on SDS-PAGE only a single protein band could be observed (Fig. 10, channel a) which had the same Rm as conventionally purified cationic peroxidase (Fig. 10, channel b). This decrease in RZ value may be due to the loss of heme from peroxidase, by the high salt concentration, used to detach peroxidase fracton from the IgGs. A similar loss of heme from a hemoprotein, with high salt concentration has been reported for cytochrome oxidase (Silvestrini _et al_., 1983).

Figure 10. SDS-PAGE (8.75%) of the (a) 8 μg of immunoaffinity purified peroxidase from unfractionated medium proteins, peak 2 of Fig. 9B; (b) 10 μg of CMC purified cationic peroxidase; (c) 50 μg of unfractionated medium proteins; (d) molecular weight standards. Phosphorylase b (94 kD); Bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); soybean trypsin inhibitor (20 kD) and α-lactalbumin (16.4 kD).

c
d

CHAPTER 5

LOCALIZATION OF THE BIOSYNTHETIC PATHWAY OF CATIONIC PEROXIDASE IN PEANUT CELLS IN CULTURE

5.1 Introduction

The results in Chapter 3 show that cationic peroxidase was secreted both by peanut cells in culture as well as in peanut plant. In this chapter, the object was to determine whether cationic peroxidase was present in the microsomal fraction, where most of the proteins destined for export are processed. In addition, attempts were made to examine by radioimmunoassays whether it was present or associated with other cellular organelles. This examination is important because peroxidase had been reported to be associated with almost all cellular organelles. (Table 1). The other relevant point is that for the complete extraction of peroxidase activity from tissues and cells, a high salt extraction has to follow the low ionic buffer extraction (Lee, 1973; Kossatz and van Huystee, 1976; van Huystee and Lobarzewski, 1982). The peroxidase extracted by the high salt buffer is localized in the cell walls (Lee, 1973), but the intracellular origin and processing site of this secreted peroxidase is not yet known (Penel et al., 1984). These and other points investigated by immunochemical techniques are examined in this chapter.

5.2 Material and Methods

5.2.1 Extraction of organelles

The cells (4-6 days old) were collected by filtering on a Whatman No. 1 filter paper under vacuum. These cells were then washed with 2 volumes of cold distilled water. The cells were broken by mortar and a pestle in 2 volumes of buffer [(300 mM mannitol, 5 mM EDTA, 0.05% cysteine, 30 mM MOPS, pH 7.5) (Moore and Beevers, 1974)]. The slurry was filtered through Nitex (22 μ) to remove any unbroken cells and large pieces of cell wall. The filtrate was subjected to differential centrifugation to separate crude preparation of amyloplast, mitochondria, nuclei and microsomes (Fig. 11). The crude preparations were purified as described below.

5.2.1.1 Amyloplasts

The method of Mills and Joy (1980) for the rapid isolation of chloroplasts was followed. The amyloplast pellet was suspended in 3 ml of buffer (330 mM sorbitol, 50 mM Tricine-KOH, pH 7.9; 2 mM EDTA and 1 mM $MgCl_2$). The 3 ml suspension was layered over 10 ml, 40% v/v percol, 330 mM sorbitol, 50 mM Tricine-KOH, pH 7.9. This was centrifuged at 2,500 g for 1 minute in a swinging bucket rotor. The resulting white pellet was later identified as amyloplasts (Section 5.2.3.5). However, most of the amyloplasts were broken (Section 5.3.1).

5.2.1.2 Nuclei

The method of Price (1979) was used to purify the crude nuclei. The nuclear pellet was resuspended in 2 ml of 0.05 M Tris-maleate pH

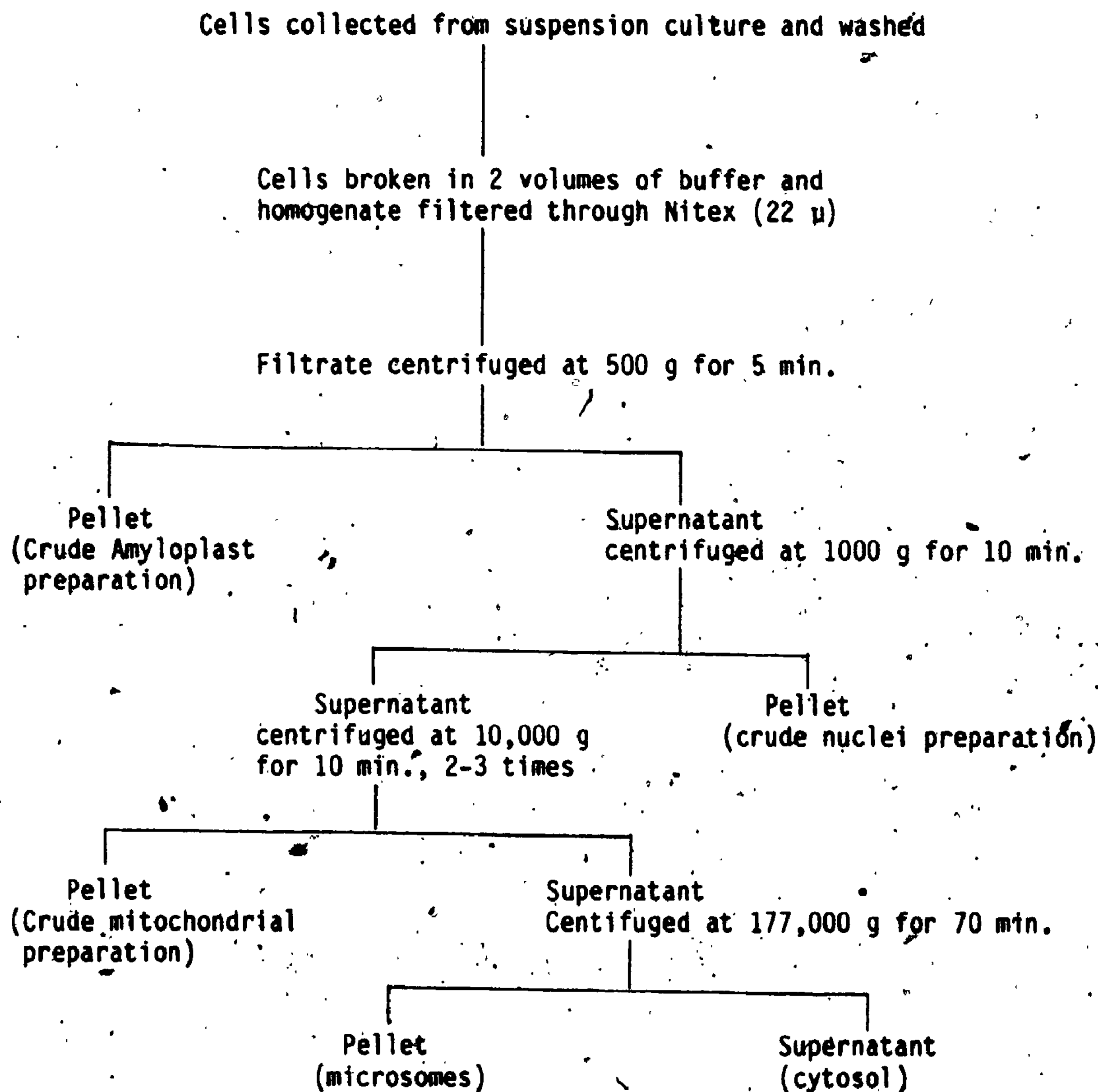


Figure 11. Flow sheet for the fractionation of cell homogenate into crude organellar preparation from cultured peanut cells.

6.6; 5 mM $CaCl_2$, 5 mM Mg acetate, 5 mM β-mercaptoethanol containing 20% glycerol, 6% w/v PVP (10,000 molecular weight). This was layered over 30% w/v sucrose and 30% v/v glycerol and centrifuged at 15,000 g for ten minutes. The white band at the interphase was later identified as nuclei (Section 5.2.3.4).

5.2.1.3 Mitochondria

The crude mitochondrial pellet was purified by the method of Bonner Jr. (1967). The mitochondrial pellet was suspended in 5 ml of 0.3 M mannitol, 5 mM EDTA, 30 mM MOPS, pH 7.5 and centrifuged at 250 g for 10 minutes. The pellet was usually nuclei and used to enrich the crude nuclear fraction. The supernatant was centrifuged at 9,000 g for 15 minutes. The supernatant so obtained along with the fluffy greenish layer on the top of the brown pellet was discarded. The brown pellet was washed in the mitochondrial buffer one or two more times to further purify it. All centrifugation were carried out in a swinging bucket rotor.

5.2.1.4 Microsomes

The 177,000 g pellet was suspended once again in the extraction medium (section 5.2.1) and centrifuged at 177,000 g for 60 minutes in a type 40 rotor. The pellet so obtained was used as microsomal pellet.

5.2.2 Isolation of polysomes

Polysomes were isolated from peanut cells in culture, essentially according to the method of Beachy et al. (1978) and Stephan and van

Huystee (1980) with slight modifications. Ten minutes before the separation of 10 g of cells from the medium, 40 ug ml^{-1} cycloheximide was added to the 50 ml of cell suspension cultures. After filtration the cells were frozen in liquid nitrogen. These frozen cells were homogenized in the extraction buffer (1:3 weight/volume) which comprised 150 mM Tris-Cl (pH 8.0), 200 mM sucrose, 50 mM KCl, 20 mM Mg acetate, 10 mM DTT and 25 mM EGTA (pH 8.5) in a glass homogeniser. The cell homogenate was filtered through 22 u Nitex to remove any unbroken cells and large pieces of cell wall. The filtrate was centrifuged at 1,000 g for 10 minutes in SS34 rotor. The pellet was discarded and the supernatant centrifuged at 66,000 g for 30 minutes in a type 30 rotor. The 66,000 g supernatant was used for the isolation of free polysomes. The 66,000 g pellet was treated with 1% Triton X-100 and 1% DOC in extraction buffer for 30 minutes at room temperature to liberate the membrane bound polysomes. The 66,000 g pellet was dispersed gently with a round edged glass rod but vortexing was avoided. The 66,000 g supernatant (free polysomes) as well as the membrane liberated polysomes were layered over a 2 ml of 1.5 M sucrose cushion in extraction buffer (see above in this section), in a type 40 rotor centrifuge tubes. This was centrifuged at 130,000 g for 4 hours, to obtain a pellet. The pellets were rinsed in sterile distilled water and resuspended in 200 to 300 µl of resuspension buffer (50 mM Tris-acetate, pH 8.5; 20 mM KCl; 10 mM Mg acetate, 10 mM DTT and 25 mM EGTA).

Analysis of the population of the free and membrane liberated polysomes was carried out following layering of one or two absorbance units at 260 nm of polysomes over a continuous 12.5 to 50% (w/v) sucrose

gradient prepared in resuspension buffer. Following centrifugation at 105,000 g in a SW 41 rotor for 90 minutes, the gradient was monitored by an ISCO-UA 5 monitor set at 254 nm.

5.2.3 Assays for markers for organelles

5.2.3.1 Cytochrome 'c' oxidase (E.C.1.9.3.1) is a marker for mitochondria (Quail, 1979).

To 0.1 ml of the organellar preparation (25-50 μg protein) in 50 mM phosphate buffer, (pH 7.5) was added 0.1 ml of 0.3% (v/v) Triton-X 100 and then shaken for 30 to 45 seconds. To this organellar preparation was added 2.7 ml of 50 mM phosphate buffer (pH 7.5) and 0.1 ml of reduced cytochrome c (0.45 mM) in phosphate buffer. The final reaction volume was 3 ml. The change in A_{550} nm was recorded over 1 to 2 minutes. The rate of change in absorbance at 550 nm was calculated from the linear portion of the line. The concentration of cytochrome 'c' oxidized was calculated as follows.

$$\frac{\Delta A_{550}\ \text{nm, min}^{-1}}{29.5}$$

E_{mM} of cytochrome 'c' is 29.5 (from manufacturer).

5.2.3.2 IDP phosphatase (E.C.3.2.2.2) is a marker for the microsomal fraction (Quail, 1979).

To 0.9 ml of IDP (3.33 mM dissolved in 36 mM Tris-MES buffer pH 7.5 containing 1.67 mM $MgSO_4$, 55.5 mM KCl was added 0.1 ml of the membrane

fraction (25 to 50 μg protein). The reaction was allowed to proceed for 30 minutes at 30 to 35°C. Two ml of ice cold 1% (w/v) of Ammonium molybdate was added to stop the reaction. The inorganic phosphate released was determined directly by adding 4 ml of reducing agent (Fiske and Subba Row, 1925). This was mixed thoroughly by vortexing and then incubated at room temperature for 35 minutes. Absorbance of each sample was measured at 660 nm. The exact amount of inorganic phosphate (Pi) released was calculated from a standard curve using Pi as a standard.

5.2.3.3 Glucose-6-phosphatase (E.C.3.1.3.9), is a marker for microsomal fraction (Rip et al., 1981).

To 5 to 15 μl (25 to 50 μg protein) of organellar preparation was added 3.6 mM imidazole-HCl (pH 6.5) containing 2 mM glucose-6-phosphate 4 mM $MgCl_2$ to a final volume of 3 ml (Rip and Cherry, 1976). Reactions were carried on for 20 minutes at 37°C and terminated by addition of cold-perchloric acid to a concentration 5%. Inorganic phosphate liberated was calculated in 1 ml of the reaction mixture as described in section 5.2.3.2.

5.2.3.4 Determination of RNA and DNA

RNA was also used as a marker for microsomes, while DNA was used for nuclei. Aliquots of all organellar fractions were sequentially extracted for RNA and DNA essentially according to the procedure reported by Rip et al. (1981). Nucleic acids and proteins in extracts were precipitated by adding cold perchloric acid to concentration of 5% and were pelleted by centrifugation. The precipitate was washed twice with 5 ml aliquots of 1.5% perchloric acid.

Pellets obtained from PCA precipitation were taken up in 5 ml of 0.3 N NaOH and RNA was hydrolyzed by incubation at 37°C for 2 hr. Each incubation mixture was then adjusted to 5% perchloric acid, chilled and centrifuged. The resulting pellets were washed twice with 5 ml aliquots of cold 1.5% perchloric acid and the washes were added to the initial supernatant. Aliquots of the combined supernatant were used to determine RNA.

Pellets containing DNA and protein were suspended in 5 ml of 1.5% perchloric acid and incubated at 90°C for 45 minutes to hydrolyze DNA. Samples were acidified to 5% perchloric acid and protein precipitates were collected by centrifugation. The protein pellet was washed twice with cold 1.5% perchloric acid and the supernatant pooled with the initial DNA hydrolyzate.DNA and RNA contents were calculated from the absorption at 260 nm, by the relationship given by Maniatis et al. (1982). One absorbance unit at 260 nm for DNA is 50 μg/ml and for RNA is 40 μg/ml.

Protein was calculated by the method of Lowry et al. (1951).

5.2.3.5 Starch

Starch, used as a marker for amyloplast was qualitatively tested with the iodine test (van Huystee and Turcon, 1973).

5.2.4 Preparation of samples for electron microscopy

For transmission electron microscopy the procedure was the same as reported by McKeen and Svircev (1981). The organellar pellets were fixed for 30 minutes in fresh cold mixture of equal parts of 4%

glutaraldehyde and 2% osmium tetroxide buffered in 0.1 M sodium cacodylate at pH 6.8. The fixed organellar pellets were then rinsed with fresh cacodylate buffer and post fixed for 30 minutes in 2% osmium tetroxide. After fixation the material was rinsed in water, stained in 5% uranyl acetate for 20 minutes at room temperature, dehydrated in graded acetone series and infiltrated with Epon-Araldite (McKeen, 1971). Sections cut with a diamond knife on a Porter-Blum ultra microtome (Sorvall MT-2), were mounted on copper grids and stained with lead citrate (Reynolds, 1963). The sections were examined on a Phillips EM 200, electron microscope.

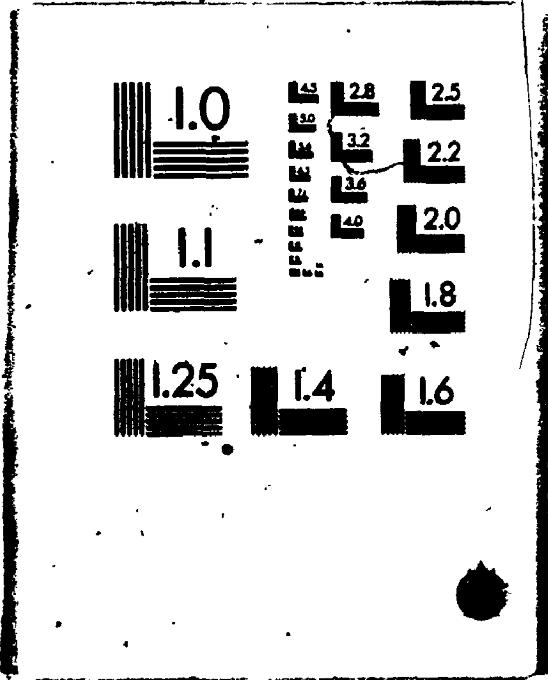
5.2.5 Optimum period for incubation of cells with ^{35}S methionine for in vivo peroxidase synthesis

To find the optimum period for incubation with ^{35}S methionine, ten ml 4 days old suspension cultures (ca ≃2 g fresh weight) were incubated separately in 5 flasks with 0.9 M Bq of ^{35}S methionine. The cells from the first flask were harvested at 0.5 hour, the remainder at 1, 2, 3 and 4 hours, respectively. The total radioactivity as well as the radioactivity in TCA precipitated proteins and immunoprecipitated peroxidase was measured in the culture medium, low ionic and high ionic cell extracts, as described earlier in Materials and Methods sections 2.9.2 and 2.9.3.

5.2.6 Subcellular localization of peroxidase in peanut cells in suspension culture

Based on the results of the above experiment, 50 ml of enriched

2



cell cultures (ca ≃ 15-20 g fresh weight) were incubated with 5 M Bq of ^{35}S methionine for 2 hours. The organelles were extracted and isolated as described in section 5.2.1. The radioactivity in the TCA precipitated proteins and immunoprecipitated peroxidase was measured for the medium, supernatant of 177,000 g as well as crude cell homogenates (Fig. 12).

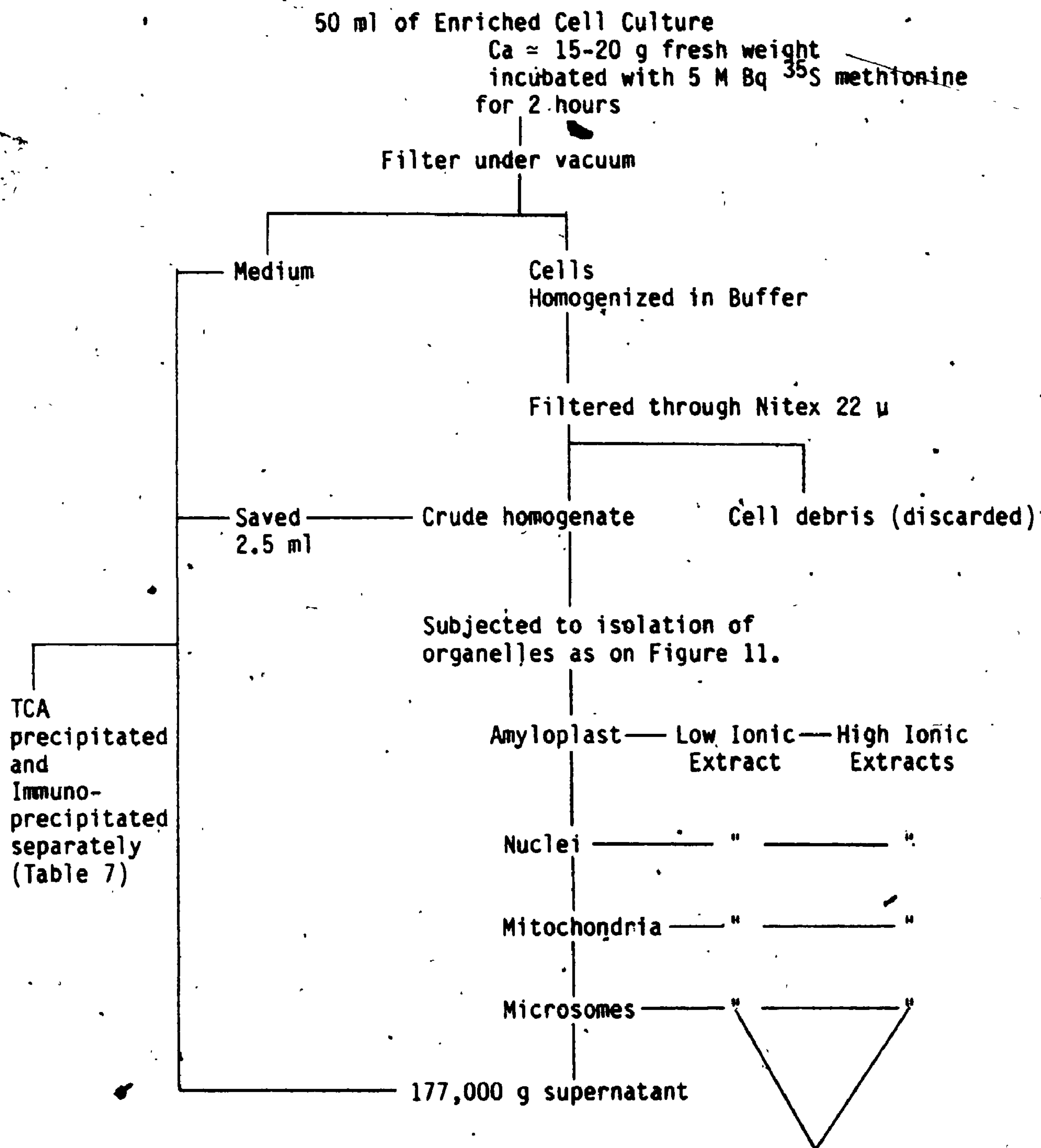
The four groups of cell organelles (Fig. 12) were first extracted with 2 mls of 0.05 M phosphate buffer, pH 7.0 (low ionic) for 30 minutes. The organelles after extraction with low ionic buffer were separated by centrifugation. Amyloplast, nuclei and mitochondria could be pelleted individually at 15,000 g or less. The microsomes had to be centrifuged at 100,000 g for 30 minutes. A second extraction with another 2 ml of low ionic buffer was done as above. Following the low ionic extraction the organelles were extracted with high ionic (HI) buffer (0.05 M phosphate buffer and 0.8 M Kcl) for 30 minutes. The organelles were separated from the extract as described for low ionic extraction. A brief outline of the experiment is described in Fig. 12.

Three aliquots of 0.1 ml and 0.5 ml were precipitated, respectively, for total proteins by TCA as well as peroxidase by immunoprecipitation with antiperoxidase IgGs, as described in section 2.9.2 and 2.9.3. Radioactivity in both these precipitates was measured separately.

5.2.7 Localization of peroxidase synthesis in polysomes - an immunochemical approach

Fifty ml of enriched cell cultures (ca ≃ 15 to 20 g fresh weight of cells) at 2, 4, 6 and 8 days of culture, were incubated with 5 M Bq of

Figure 12. Flowsheet for the experimental plan to study the intracellular localization of peroxidase and origin of high and low ionic extracts of peroxidase in peanut cells in culture.



50 ml of Enriched Cell Culture
Ca ≈ 15-20 g fresh weight
incubated with 5 M Bq 35S methionine
for 2 hours
Filter under vacuum
Medium
Cells
Homogenized in Buffer
Filtered through Nitex 22 μ
Saved
2.5 ml
Crude homogenate
Cell debris (discarded)
Subjected to isolation of
organelles as on Figure 11.
TCA
precipitated
and
Immuno-
precipitated
separately
(Table 7)
Amyloplast
Low Ionic
Extract
High Ionic
Extracts
Nuclei
Mitochondria
Microsomes
177,000 g supernatant
Low and high ionic
extracts from each
organelle were
TCA precipitated and
immunoprecipitated
(Table 8).

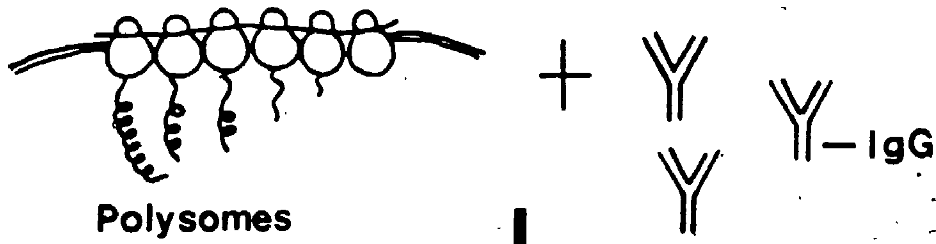
^{35}S methionine for 2 hours (Section 2.9.1).

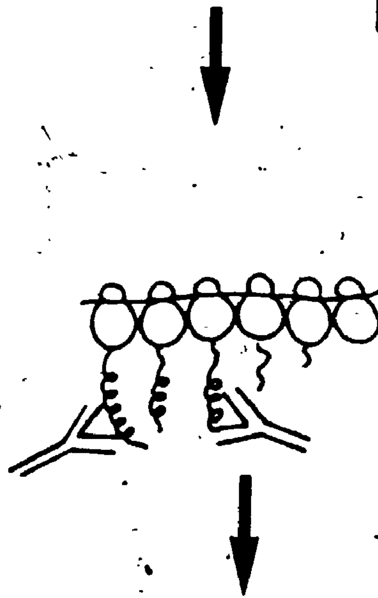
After 2 hours of incubation, the cells were filtered under vacuum and extracted for polysomes (both free and membrane bound) as described above in section 5.2.2. Three aliquots (1, 2 and 3x in terms of concentration of RNA) of polysomes were mixed with specific IgGs in 0.1 M phosphate buffer pH 7.0 in a ratio of 6:1 (wt/wt. RNA/protein) as suggested by Shapiro and Young (1981). A control with non-specific IgGs (section 4.2.3) in a similar manner was also run to check for non-specific binding. The polysomes and IgGs were allowed to react overnight at 5-10°C. Next day 50 µl of 10 percent formalin fixed <u>Staphylococcus</u> <u>aureus</u> protein A (immunoprecipitin) was gently mixed with polysome - nascent peroxidase - IgG and left to react with the Fc (class specific) portion of IgG (Fig. 13) for 30 minutes at room temperature. This immunoprecipitated complex (Fig. 13) was treated as described in section 2.9.3.

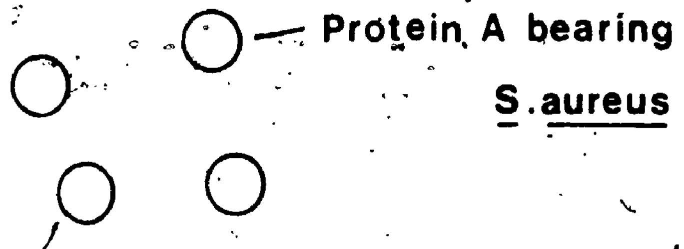
The washed immunoprecipitated complex was finally dissolved in 5 ml aquasol. The radioactivity due to either the nascent peroxidase or proteins (non-specific binding) was measured for polysomes associated with peroxidase synthesis. The radioactivity obtained with the non-specific IgGs was subtracted from that of the mono-specific IgGs to account for the non-specific binding.

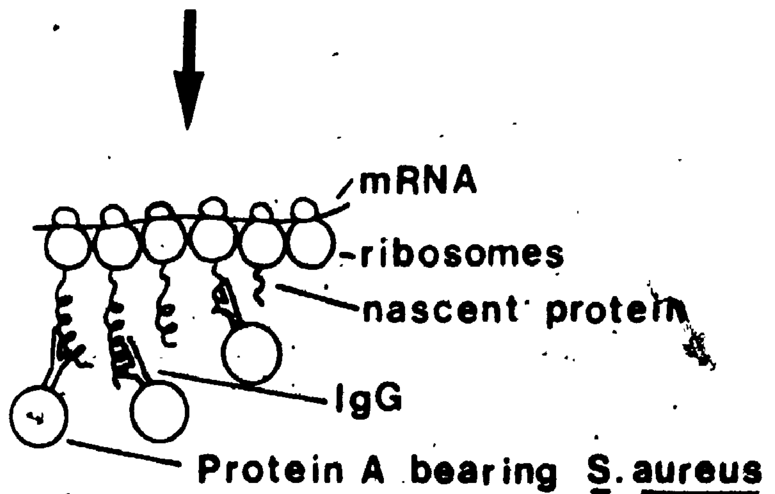
The radioactivity for polysomes associated with total protein synthesis was measured by the method of Mans and Novelli (1961) as described in section 2.9.2.

Figure 13. Immunoprecipitation of peroxidase synthesizing polysomes. Schematic (not to scale) drawing to show the formation of polysome-nascent peroxidase (protein) - IgG - Protein A - _S. aureus_ complex.


Polysomes
IgG




Protein A bearing
S.aureus

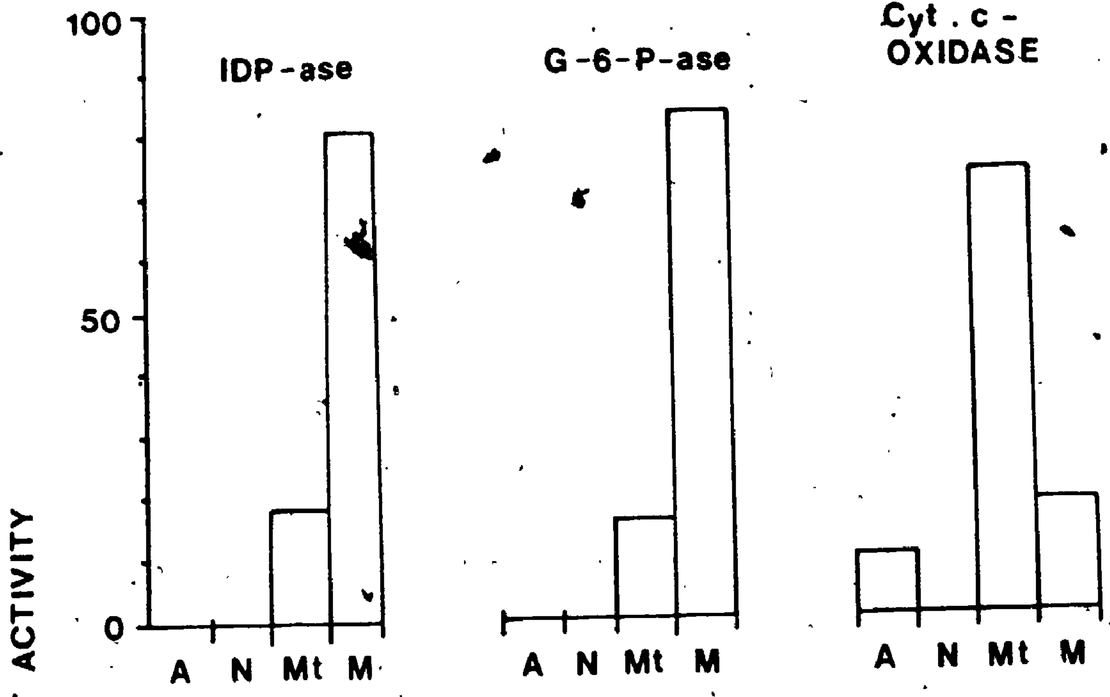

mRNA
ribosomes
nascent protein
IgG
Protein A bearing S.aureus

5.3 Results and Discussion

5.3.1 Isolation of cell organelles from cultured cells

The results presented in Figs. 14 and 15 to 17 showed that amyloplasts, mitochondria, nuclei and microsomes had been isolated from peanut cells in suspension culture. A relatively pure preparation of these organelles was obtained as checked by high percent of respective marker's activity (Fig. 14). The cross contamination was less than one fifth between the microsomes and mitochondria, in either fraction (Fig. 14). The same observation was supported by the electron microscopic observations of the mitochondrial and microsomal fractions (Fig. 15 and 16). At least a dozen sections were scanned before choosing a representative one. Electron micrographs for amyloplasts could not readily be obtained because the membranes were easily fractured and usually starch granules, with membranes ruptured at various places were observed (Fig. 17A). The nuclear pellet obtained was also small in volume. Electron micrographs were not taken. There have been few reports on the isolation of organelles from cultured cells. The usual approach has been to carry out an isopycnic centrifugation on sucrose density gradients and monitor the enrichment of marker enzymes in the fractions of those gradients (Moore and Beevers, 1974; Quail, 1979). Thus, the technique of differential centrifugation used in this study, is an improvement of earlier methods as indicated by high percent of S.A. of the respective markers in each fraction.

Figure 14. Marker activities in the four group of organelles, isolated from cultured peanut cells. Amyloplasts (A); nuclei (N), mitochondria (Mt) and microsomes (M). The total marker activity in all four fractions was added up to give 100 percent. The percent activity in each fraction was then calculated. This was a representative of three experiments.


100
50
0
IDP-ase
G-6-P-ase
Cyt. c -
OXIDASE
A N Mt M
A N Mt M
A N Mt M
PERCENT ACTIVITY

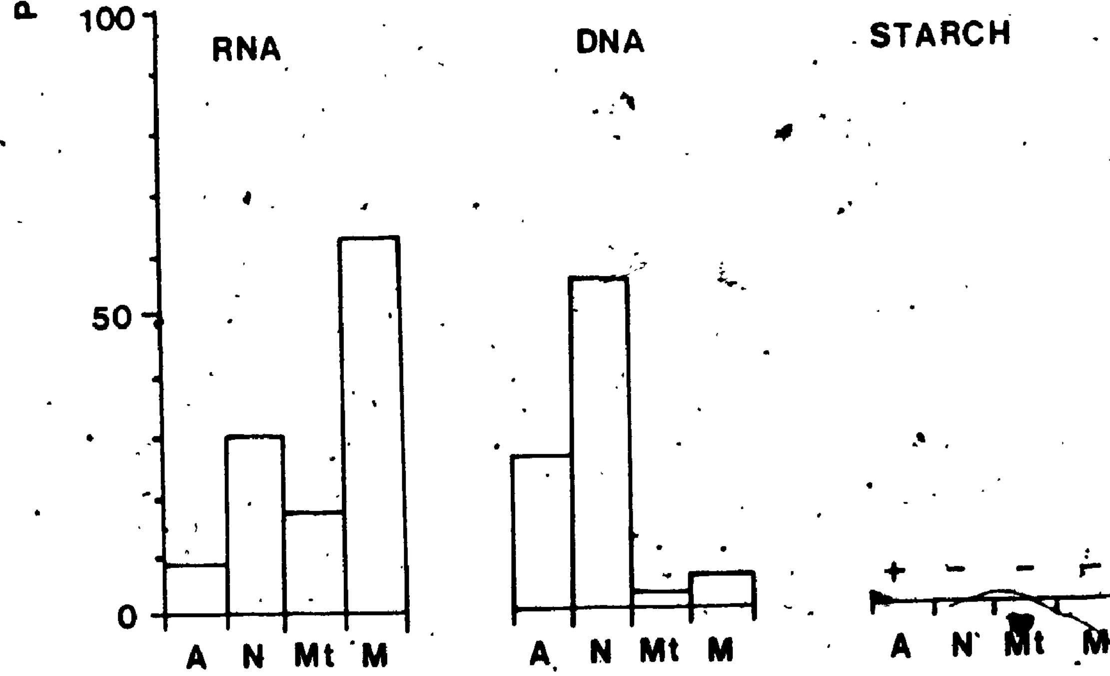
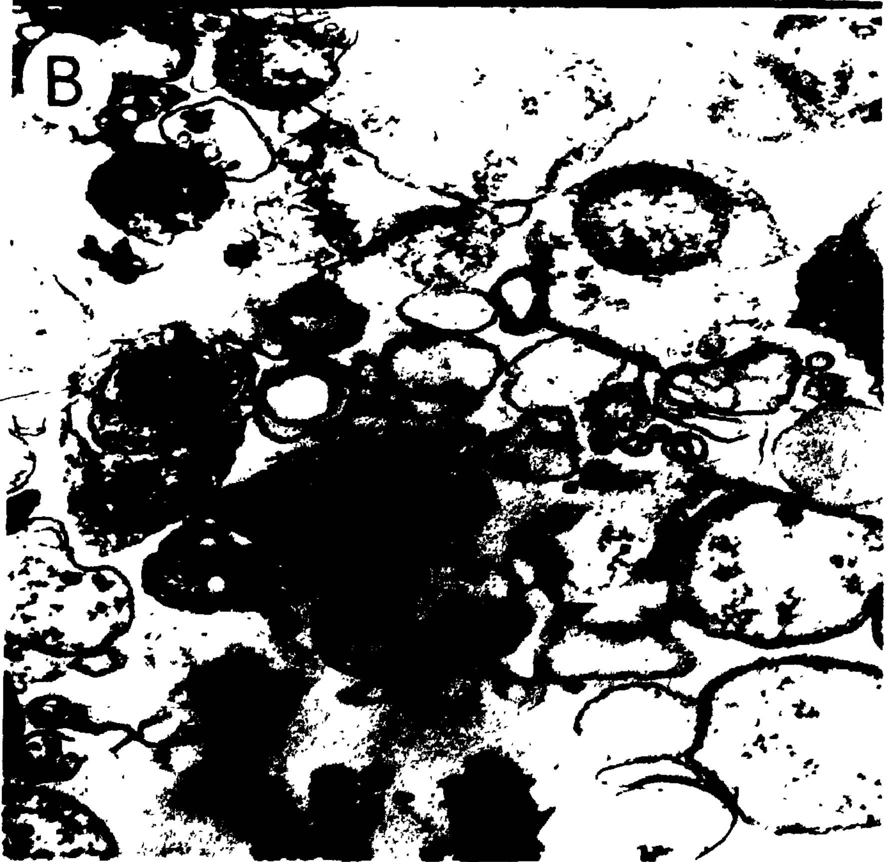

100
50
0
RNA
DNA
STARCH
+ - - -
A N Mt M
A N Mt M
A N Mt M

Figure 15. Transmission electron micrograph of mitochondria. (Magnification 37,100x). A and B were two representative sections from at least a dozen preparations.

A


B

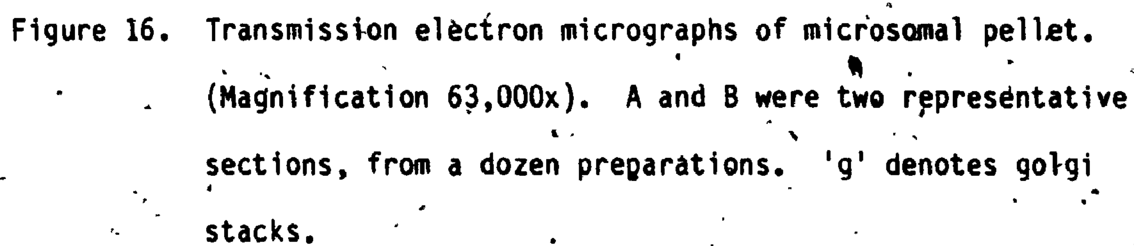
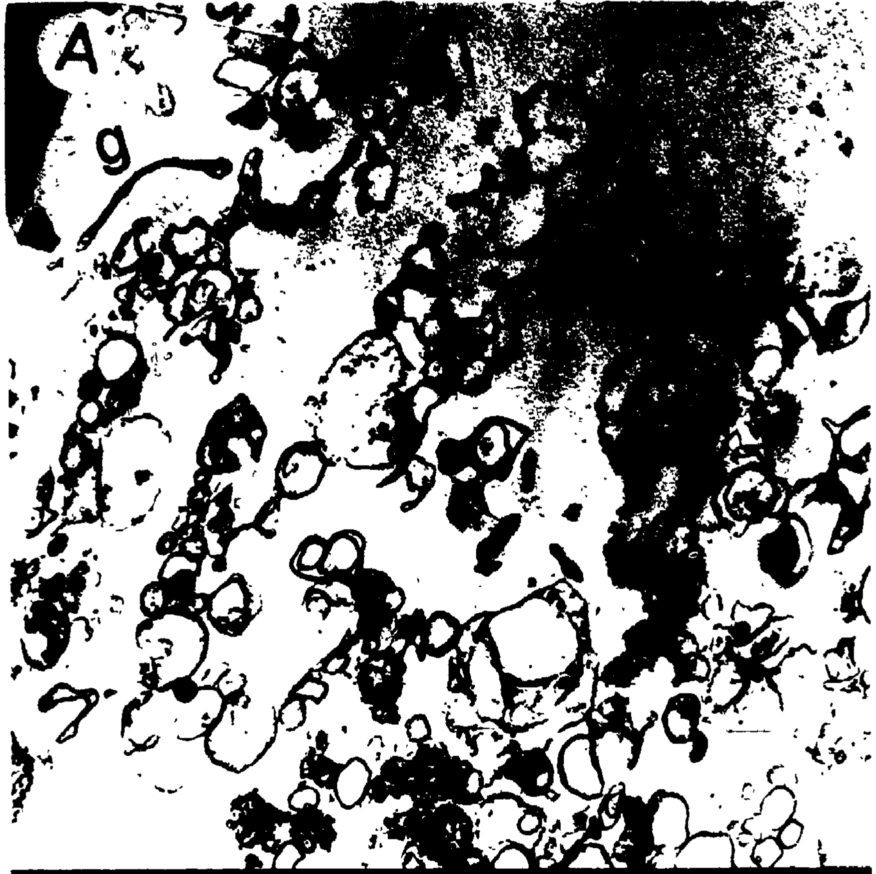


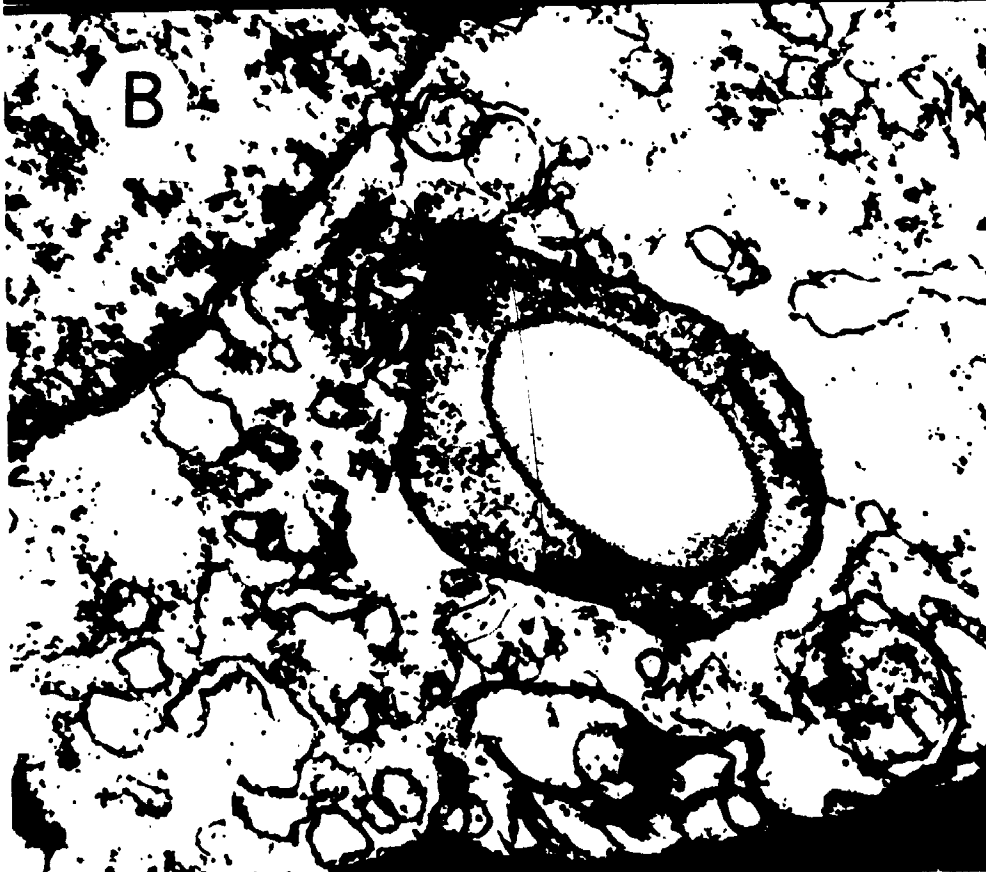
Figure 16. Transmission electron micrographs of microsomal pellet. (Magnification 63,000x). A and B were two representative sections, from a dozen preparations. 'g' denotes golgi stacks.


A
g

B
g

Figure 17. Transmission electron micrograph of amyloplasts. (A) was obtained from amyloplast pellet after isolation of amyloplast. Note the broken membrane around starch granule (Magnification 36,000x). (B) intact amyloplast obtained from sections of whole cells (Magnification 38,000x).

A


B

5.3.2 Optimal time for incubation of cells with ^{35}S methionine for maximal peroxidase synthesis

The results presented in Figs. 18 and 19 suggested that 2 hours was the optimal time of inqubation in terms of maximal peroxidase synthesis, with the conditions used here. The amount of total radioactivity being incorporated into the cells i.e. lost from the medium increased up to two hours. After two hours the radioactivity almost remained the same in the medium, probably due to the release of proteins from the cell (Fig. 18A). These data agreed well with the amount of total radioactivity observed to accumulate in the low ionic extract (Fig. 18A), where it increased for two hours but declined slightly after two hours. However, in the case of high ionic extract, the increase of radioactivity incorporated did not change (Fig. 18A). There was also a gradual increase in the release of peroxidase into the medium, as observed by radioimmuno assays (Fig. 18B). Maximal rates of peroxidase synthesis were observed at 2 hours in the case of high ionic buffer extracts (Fig. 19B). The radioactive peroxidase in the low ionic extracts was highest at 3 hour (Fig. 19A). Total protein synthesis showed a gradual increase in both low and high ionic extracts (Fig. 19A and B). These data on total protein synthesis agreed well with the results of Verma and van Huystee (1970). Using ^{14}C leucine they also observed a gradual increase up to 3 hours in total protein synthesis in cultured peanut cells. Maximal peroxidase synthesis in this study was observed at 2 hours, hence 2 hours of incubation was used in further studies.

Figure 18. A - Total radioactivity amongst the components of the culture; viz the medium, low ionic and high ionic cell extracts of peanut cells in culture. The total radioactivity in the three fractions was added up to give 100 percent. The percent radioactivity in each componenet was then calculated in relation to this total radioactivity at each sampling time.

B - Release of cationic peroxidase into the medium of cultured cells. Five aliquots of 0.5 ml were taken for immunoprecipitation, with antiperoxidase serum, as described in section 2.9.4. The mean radioactivity determined, was used to calculate for the total peroxidase in the total volume of medium.

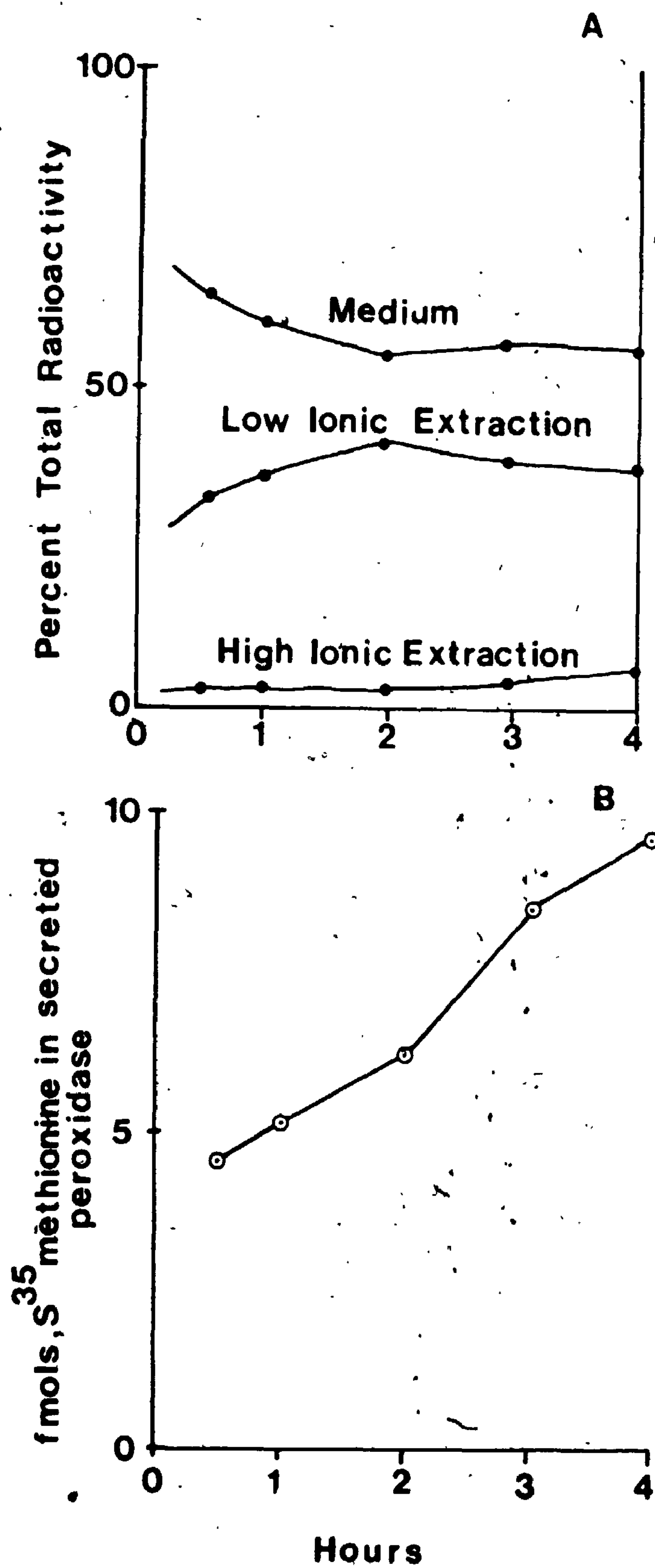
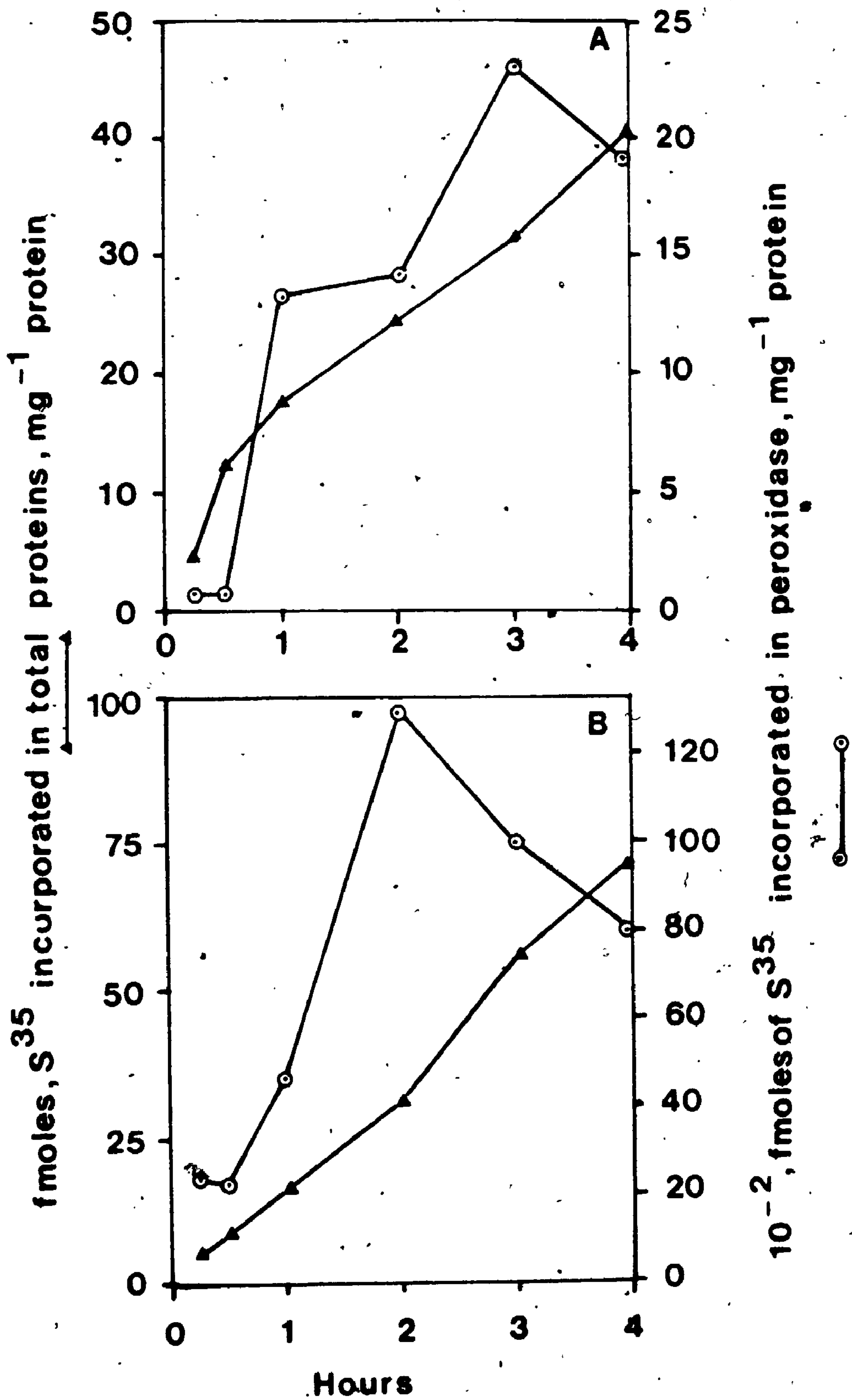

A
100
50
0
Percent Total Radioactivity
Medium
Low Ionic Extraction
High Ionic Extraction
0
1
2
3
4
B
10
5
0
fmols, S35 methionine in secreted peroxidase
0
1
2
3
4
Hours

Figure 19. Peroxidase (⊙——⊙) and protein (▲—▲) synthesis in the low (A) and high (B) ionic extracts of cultured peanut cells.

Two g fresh weight of cells were sequentially extracted at different time intervals in the low and high ionic buffer as described in section 5.2.5. Three aliquots of 0.1 and 0.5 ml were TCA and immunoprecipitated for total protein and peroxidase synthesis respectively. Results were calculated as f mole ^{35}S methionine incorporated mg^{-1} protein.


A
B
fmoles, S35 incorporated in total proteins, mg-1 protein
10-2, fmoles of S35 incorporated in peroxidase, mg-1 protein
Hours

5.3.3 The overall percent of peroxidase synthesis to total protein synthesis

The results presented in Table 7 showed that the cationic peroxidase represented about 2.6% of the total proteins synthesized which was similar to that reported in Chapter 3, section 3.3.3. The cationic peroxidase also formed one sixth of the total proteins in the medium, as reported in Chapter 3, section 3.3.1. The interesting point was that the cationic peroxidase formed only 0.8% of the total proteins in the soluble phase of cells (i.e. 177,000 g supernatant). This agreed well with the results of low ionic extractions as reported by van Huystee and Lobarzewski (1982).

5.3.4 Association of peroxidase with cell organelles

The results presented in Table 8 showed that a ten-fold higher radioactivity was associated with peroxidase in the microsomal fractions in the high ionic over low ionic extracts. Conversely a nearly uniform radioactivity, less than 1,000 cpm, was associated with all the organelles in the low ionic as well as high ionic extracts. This could be interpreted in two ways. One was that peroxidase was associated with all organelles as reported by other workers (Table 1) or conversely, these 1,000 cpms may be taken as background and may represent a non-specific binding.

Table 7. Distribution of newly synthesized proteins and peroxidase in fractions of cultured peanut cells.*

Fraction	cpm in Proteins (TCA precipitate)	cpm in Peroxidase (immuno-precipitate)	Percentage of peroxidase to total protein
1. Crude homogenate	15×10^6	0.4×10^6	2.6
2. Medium (secreted)	1.8×10^6	0.3×10^6	16.6
3. Supernatant 177,000 g	8×10^6	0.06×10^6	0.8

* Data represents at least 3 independent experiments with identical trends.

Table 8. Subcellular distribution of newly synthesized proteins and peroxidase in cultured peanut cells.*

Organelles (Total fraction)	cpm in		Percentage of peroxidase to total proteins
	Proteins (TCA precipitate)	Peroxidase (immuno-precipitate)	
A. Low Ionic Extraction			
Amyloplasts	7.9×10^3	0.5×10^3	7
Nuclei	9.6×10^3	0.2×10^3	2
Mitochondria	42×10^3	0.9×10^3	2
Microsomes	63×20^3	0.9×10^3	1.5
B. High Ionic Extraction			
Amyloplasts	31×10^3	0.7×10^3	2
Nuclei	1.8×10^3	0.09×10^3	5
Mitochondria	33×10^3	1×10^3	3
Microsomes	75×10^3	11×10^3	15

* Data represents at least 3 independent experiments with identical trends.

5.3.5 The intracellular origin of the high ionic extraction of peroxidase

The results presented in Table 8B clearly showed that the major proportion of peroxidase extracted with the high salt buffer, originated from the microsomal fraction. Fifteen percent of the total proteins extracted with the high ionic buffer was peroxidase, as compared to only 1.5 percent in the low ionic buffer (Table 8). It may be noted here that almost the same amount of radioactivity was associated with TCA precipitated proteins, in the low and high ionic extracts of mitochondria and microsomes. The mitochondria served as a good control for microsomes. The reason for the four fold increase of extraction of TCA precipitated proteins in the amyloplast, was not understood. The only explanation that could be offered was that there might have been contamination of the small cell wall fragments of equal density to that of amyloplasts, which might have given rise to this increased radioactivity (Table 8B). This contamination was partly shown by the results of electron micrographs (Fig. 17A), where some fragments may be seen. Preliminary data from monensin treatment of peanut cells gave support to the hypothesis of the nature of high ionic extract (Appendix 1).

5.3.6 In vivo localization of peroxidase synthesizing polysomes

The observations in the preceding sections in this chapter showed the association of peroxidase with microsomes. Polysomes are the site of synthesis of the proteins. Peroxidase has been reported to be synthesized on membrane bound polysomes (van Huystee, 1978; Stephan and

van Huystee, 1981). In this section it was shown that the in vivo ratio of peroxidase to total protein synthesis was higher on the membrane bound polysomes than on the free polysomes by employing immunochemical techniques.

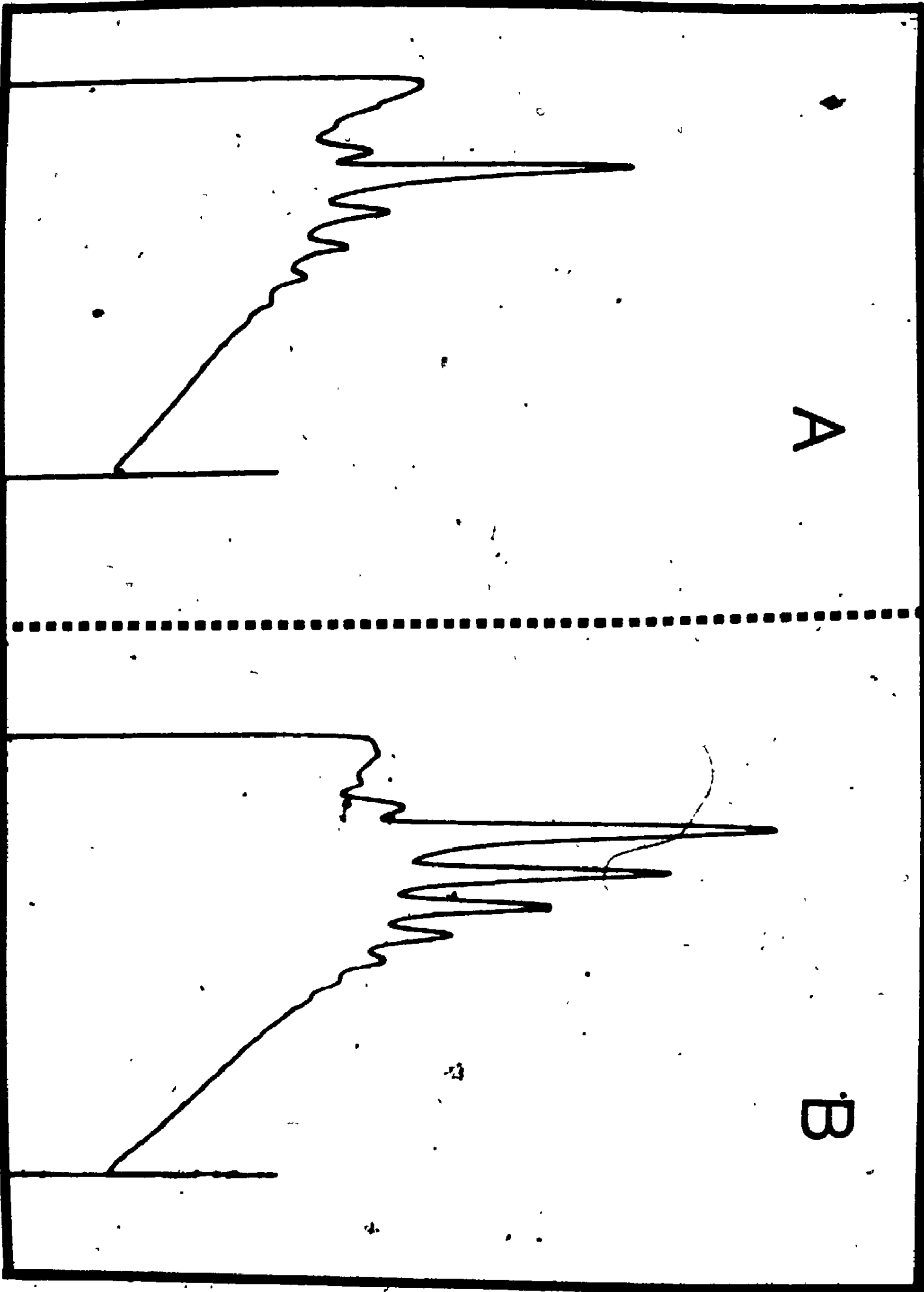
5.3.6.1 Isolation of polysomes

The results (Fig. 20) showed that polysomes could be isolated from peanut cells in culture. As far as the class characterization was considered there were more polysomes (4-5, ribosomes) in the membrane liberated than in the free polysomes (Fig. 20). Different times of centrifugation at 66,000 g revealed that with increasing time, more membrane bound polysomes could be pelleted based on RNA content (Table 9). Since there is no unanimity in the literature as to the percentage of free to membrane bound polysomes in plants, the centrifugation time of 30 minutes was selected for further experiments. The time of centrifugation for 30 minutes gave a reasonable yield of polysomes and the ratio did not deviate considerably from the only other report of polysome from plant cells (Pfisterer and Kloppstech, 1976). De Robertis and De Robertis (1980) have suggested a ratio of 15 to 25% for membrane bound to free polysomes in maize mesophyll cells which is nearly identical to that of 30 minutes of centrifugation for peanut polysomes.

5.3.6.2 Percentage of free to membrane liberated polysomes during culture cycle

The percentage of free-to membrane-liberated polysomes, as determined by RNA content in these polysomes, did not change over the

Figure 20. Class characterization of the free (A) and membrane liberated (B) polysomes, from cultured peanut cells. One absorbance unit at 260 nm was gently layered over a 12.5 to 50 percent continuous sucrose gradient. After centrifugation the gradient was fractionated at a flow rate of 2 ml minute^{-1} and chart speed was 60 cms, hour^{-1}. The ISCO-monitor was set at 254 nm.



A_{254} nm
Top
Bottom
A
B

Table 9. Effect of centrifugation time* on the relative recovery of free- and membrane-bound polysomes in terms of RNA content.**

Time	Percent of RNA in free to membrane bound
15 min	88:12
30 min	69:31
60 min	40:60

* Time of centrifugation at 66,000 g to separate free- and membrane-bound polysomes.

** Free- and membrane-bound polysomes were recovered from 66,000 g pellet and supernatant, respectively.

period of days studied (Table 10). This was in agreement with the results reported by Stephan and van Huystee (1980), who used the approach of Payne and Boulter (1969). Although Payne and Boulter (1969) did find an increase in the ratio of free to membrane liberated polysomes during the growth cycle of bean. There are few reports in literature, which support this hypothesis (Stephan and van Huystee, 1980).

5.3.6.3 Association of peroxidase with polysomes

Polysomes with nascent peroxidase could be immunoprecipitated on all the days of culture period studied (Table 11). This indicated the continued synthesis of peroxidase during the culture cycle. The percent of peroxidase to total protein synthesis was three to four fold higher in the membrane liberated than free polysome (Table 11). This suggested that more peroxidase was synthesized on the membrane bound polysomes than on free polysomes (Table 11).

The technique of immunoprecipitation of polysomes was used in this research to show the association of peroxidase with polysomes, but could profitably be used by subsequent workers for detailed studies on peroxidase regulation by mRNA isolation (Maurer, 1980; Sharpiro and Young, 1981; Kraus and Rosenberg, 1982).

The results obtained by *in vivo* immunoprecipitation were fully in agreement with the results of *in vitro* synthesis of peroxidase reported by Stephan and van Huystee (1981), where they found two fold higher peroxidase synthesis by the membrane liberated polysomes than the free polysomes. However, the difference might have been more amplified as in

Table 10. Percentage of free-to membrane bound-polysomes* during active growth period in peanut cells in culture.

Days of culture	Fresh weight of cells for extraction	Percentage of free-to bound polysomes
2	10 g	77:23
4	10 g	74:26
6	10 g	71:29
8	25 g	79:21

* The concentration of polysomes was based on the amount of RNA determined by measurement of absorbance at 254 nm (see Appendix 2, Table 1).

Table 11. Percentage of polysomes associated with peroxidase* and protein synthesis during active growth of peanut cells in culture.

Days of culture	Percent of polysomes associated with peroxidase to that with protein synthesis**		
	Free	Bound	Total***
2	1.2	16.4	7.3
4	3.6	13.8	6.5
6	4.8	17.5	8.4
8	5.4	15.8	7.8

* cpm in immunoprecipitate (specific IgGs) - cpm in immunoprecipitate (non-specific IgGs), per mg RNA

** Polysomes associated with peroxidase to that of protein synthesis

$$= \frac{\text{cpm in immunoprecipitate}}{\text{cpm in TCA precipitate}} \times 100$$

*** cpm in immunoprecipitate and TCA precipitate for the free- and membrane-bound polysomes taken together. Percentage of total polysomes associated with peroxidase to that of protein synthesis

$$= \frac{\text{cpm in immunoprecipitate (free + membrane liberated polysomes)}}{\text{cpm in TCA precipitate (free + membrane liberated polysomes)}}$$

Average radioactivity of peroxidase associated with polysomes was 5×10^3 cpms, mg^{-1} RNA. (See Appendix 2, Tables 2 to 4)

the present case, if their centrifugation for separation of free and membrane liberated polysomes would have been at a higher centrifugal force (Stephan and van Huystee, 1981). Penon et al. (1970) also reported that two basic and one anionic peroxidase are associated with lentil root ribosomes. Furthermore, they also pointed out that treatment of lentil roots with IAA, stimulates the de novo synthesis of the basic peroxidase (cationic) isoenzymes but not the acidic (anionic) peroxidase. The significance of this will be discussed in a following chapter.

The results obtained in this chapter were in agreement with the secretory nature of peroxidase. Most of the secretory proteins are synthesized on membrane bound polysomes and processed (glycosylation/trimming) in the Golgi apparatus (van Huystee, 1978; Hall et al., 1982 and references cited on pp. 213-214).

CHAPTER 6

HEME MOIETY OF PEANUT PEROXIDASE

6.1 Introduction

The preceding Chapter 5 dealt with the localization of the biosynthetic pathway of cationic peroxidase in peanut cells in culture. Since peroxidase is a hemoglycoprotein, different aspects of the heme moiety had been investigated in this section. Heme forms an important part of the molecule, because of its involvement in the enzymatic catalysis associated with this molecule (Ku *et al*., 1970; Ricard and Job, 1974; Lee, 1977).

Moreover, heme could also exert an influence on the biosynthesis of the apoprotein moiety as shown for other hemoproteins. This concept could relate to the regulation of peroxidase synthesis (van Huystee and Cairns, 1980; Hamilton *et al*., 1982). Most of the reports in literature on the heme moiety of peroxidase are based on homologies with other hemoproteins (Scandalios, 1974). In Chapter 6, it was shown that peroxidase from cultured peanut cells was an ideal system to study the precursors of heme. It was also demonstrated that the heme in peroxidase was protoheme and was synthesized in the mitochondria in cultured peanut cells. In addition, the removal of heme apparently did not affect the physical structure of the molecule, but heme was nevertheless needed for catalysis.

6.2 Materials and Methods

6.2.1 Extraction of heme from peroxidase and organelles

The heme from the cationic peroxidase, immundprecipitated peroxidase or organelles was extracted by the method of Stillman and Gassman (1978) with slight modification (Chibbar and van Huystee, 1983b). The organellar pellet, immunoprecipitate, or the precipitated cationic peroxidase (obtained from isolated peroxidase in phosphate buffer by precipitating with 70% acetone) was extracted with 2 ml of chilled acidified acetone (2% v/v, HCl). The heme moiety in acetone was recovered in the supernatant by centrifugation at 14,000 g at 4°C for 10 minutes. The white pellet that remained was the apoprotein. The extraction of the apoprotein, or organelles was repeated as above to increase the yield of heme and to purify the apoprotein moiety. The two supernatants were pooled and half volume of peroxide free ether was added. To this mixture was added 2 volumes of chilled distilled water. This was shaken gently and centrifuged at 1,000 g for 5 minutes. The organic phase containing the heme was removed with a pasteur pipette. The ether was evaporated under N_2 and the dried heme was used for measurements according to the needs of the experiment.

6.2.2 Preparation of the apoprotein

Heme was cleaved from the purified cationic peroxidase (10 mg) as described in section 6.2.1. The white apoprotein pellet, left after the removal of heme was dissolved in 0.1 M phosphate buffer (pH 8.0) and brought to a final concentration of 25 μM of apoperoxidase. A fraction of this solution was dialysed extensively against 0.1 M phosphate buffer for further assays of enzymatic activities and reassociation.

6.2.3 Determination and identification of heme

6.2.3.1 Pyridine hemochrome difference spectrum

Samples containing heme were dissolved in 5 ml of alkaline pyridine (2 volumes, pyridine and 3 volumes of 0.2 M KOH) and the solution divided equally between the two cuvettes of the double beam spectrophotometer. Sodium dithionite was added to the sample cuvette. The difference spectrum of the reduced minus oxidized pyridine hemochrome was recorded. The concentration of protoheme was calculated using ΔE mM = 20.7. ΔE represents the difference in extinction between the α band at 557 nm and the trough at 540 nm between α and β peaks (Porra and Jones 1963).

6.2.3.2 High pressure liquid chromatography

The isolated heme and hemin (Eastman Kodak, N.Y.) were also identified on reverse phase Hewlett Packard 1084 B liquid chromatograph. A Lichsorb R.P.-18 column (25 cm x 4.6 mm) with 5 µm particles was used. The HPLC solvent employed was 80% ethanol (pH 3.0 adjusted with acetic acid). The running temperature was maintained at 30°C, and the flow rate was 1 ml. min^{-1}. The heme isolated from peroxidase and the hemin were dissolved in 100 µl of 0.1 M NaOH and diluted with HPLC solvent. The eluant was monitored at 402 nm.

6.2.3.3 Mass spectrometric identification

This was done on a VARIAN MAT 311A mass spectrometer. Conditions are given in the legend for mass spectrum.

6.2.4 Sedimentation analysis

The holoenzyme (2.2 $mg.ml^{-1}$) and apoperoxidase (2.0 $mg.ml^{-1}$) in a volume of 0.7 ml of 0.05 M phosphate buffer (pH 7.0) were analysed in an AN-D rotor, of Beckman Model E ultracentrifuge for sedimentation coefficient determination. The samples were centrifuged at 50,740 rpm at 20 to 25°C and photographs with the Schlieren optics were taken at 16 minute intervals. Sedimentation coefficients were then calculated according to the method of Markham (1960) and corrected to water at 20°C.

SDS-PAGE, immunodiffusion assays and enzyme assays for peroxidase and IAA-oxidase were done as described under sections 2.7, 2.8, 2.4 and 2.5 respectively.

6.2.5 Reconstitution of peroxidase

The reconstitution between apoperoxidase and hemin was carried out as described by Yonetani (1967). To the 400 µl of 25 µM solution of apoperoxidase were added varying amounts (0 to 20 µM final concentration) of 50 µM hemin in 400µl of 0.1 M sodium hydroxide. The final volume of the reaction mixture was made up to 1 ml with 0.1 M phosphate buffer (pH 8.0). After allowing 10 minutes for reconstitution the absorbance at 407 nm was recorded for each sample. In the control experiment without apoprotein, 400 µl aliquots of 0.1 M phosphate buffer were added to the increasing amounts of hemin and the absorbance at 407 nm was recorded as above.

6.2.6 Determination of precursor(s) for heme in peanut peroxidase

Aliquots (10 ml) of enriched peanut cell suspension cultures (3

days old ca ≃ 2 g fresh weight of cells) were incubated with 1-^{14}C-glycine (1.1 M Bq), 2-^{14}C-glycine (2.2 M Bq) and U-^{14}C-glutamic acid (1.1 M Bq) for 6 hours in light. The medium of each treatment was separated by filtration and four aliquots of 0.5 ml each were used for immunoprecipitation of secreted peroxidase. Peroxidase from the immunoprecipitates was cleaved into the heme and apoprotein as described in section 6.2.1. The supernatant containing the heme in ether from the four replicates of each treatment were pooled for the respective treatments to give sufficient radioactivity per 2 ml medium. Similarly the apoprotein moieties from each treatment were pooled to give corresponding data for 2 ml medium. At least three experiments were carried out.

6.2.7 Search for the site of heme synthesis in cultured peanut cells

Fifty ml of enriched cell cultures (3 days old ca ≃ 15 to 20 g fresh weight of cells) were incubated with 1.1 M Bq of ^{14}C-ALA for 4 hours. Then 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 homogenized in a pestle and mortar and the organelles (amyloplast, mitochondria and microsomes) were separated in the buffer and conditions described in section 5.2.1.

The mitochondria and amyloplast were subjected to the procedure for heme extraction as described in section 6.2.1. The labelled heme (if any) obtained in ether was dried under N_2 in scintillation vials. Following the addition of aquasol the radioactivity was measured.

6.2.8 Percentage of peroxidase to other hemoproteins in medium, cytosol and microsomes

The microsomes isolated in section 6.2.7 were sequentially extracted for proteins in low and high ionic buffers as described in section 2.9.2. The low and high ionic extracts were pooled to give the microsomal extracts. The ratio of peroxidase to total hemoproteins was determined in the microsomal extracts (this section), supernatant of 177,000 g and from the medium (in preceding section 6.2.7) by the technique of immunoprecipitation and TCA precipitation as described in sections 2.9.2 and 2.9.3.

6.3 Results and Discussion

6.3.1 Hemoproteins in the medium

The peanut culture medium contained only a few hemoproteins, as seen on SDS-PAGE gel stained for hemoproteins (Fig. 21A, channel c) and proteins (Fig. 21B, channel c). The cationic peroxidase formed the major, if not the sole hemoprotein in the medium (Figs. 21A and B). The darkly stained blue band at the front (Fig. 21A, channel b) might be due to the heme that was removed from the peroxidase, under denaturing conditions. At the same position no band was seen when the gels were stained with coomassie blue (Fig. 21B, channel b). The pre-eminence of peroxidase as the major hemoprotein was further supported by the following observation. When the cationic peroxidase was immunoprecipitated from the medium of cells incubated with ^{14}C-ALA a level of radioactivity was observed similar to that in the TCA precipitated proteins (Table 12). ALA is a committed precursor for the porphyrin biosynthesis (Granick and Beale, 1978).

Figure 21. SDS-PAGE (7.5-15% gradient gel) analysis of peroxidase and medium proteins of cultured peanut cells. Channel a - 20 μg apoperoxidase; b - 25 μg holo-enzyme; c - 100 μg medium proteins; d - markers (phosphorylase - 94 kD; bovine serum albumin 67 kD; ovalbumin 43 kD; carbonic anhydrase 30 kD; soybean trypsin inhibitor 20 kD; α-lactalbumin 14.4 kD). Figure 21A - following electrophoresis the gel was stained for hemoproteins. Figure 21B was the same gel subsequently stained for proteins with Coomassie Blue.

A

a b c d

B

a b c d

Table 12. Percentage of peroxidase to total hemoproteins* in various fractions of cultured peanut cells.**

Fraction	Total Volume (ml)	dpm x 10^3 peroxidase (immuno-precipitate)	dpm x 10^3 proteins (TCA precipitate	Percent*** peroxidase to hemo proteins
Medium	12	29.3	32.4	90
Supernatant of 177,000 g	20	28.5	132.9	21
Microsomes	2.0	6.2	8.9	70

* The hemoproteins were labelled by incubating the cells with 4-^{14}C-ALA (section 6.2.8).

** A representative of three experiments with identical trends.

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

Microsomes are known to process and, or contain the proteins destined for secretion (Chapter 5). The results of the immunoprecipitate versus TCA precipitate in Table 12, further supported the above observation. Peroxidase forms almost three quarters of the total hemoproteins in microsomes that were labelled by ^{14}C-ALA.

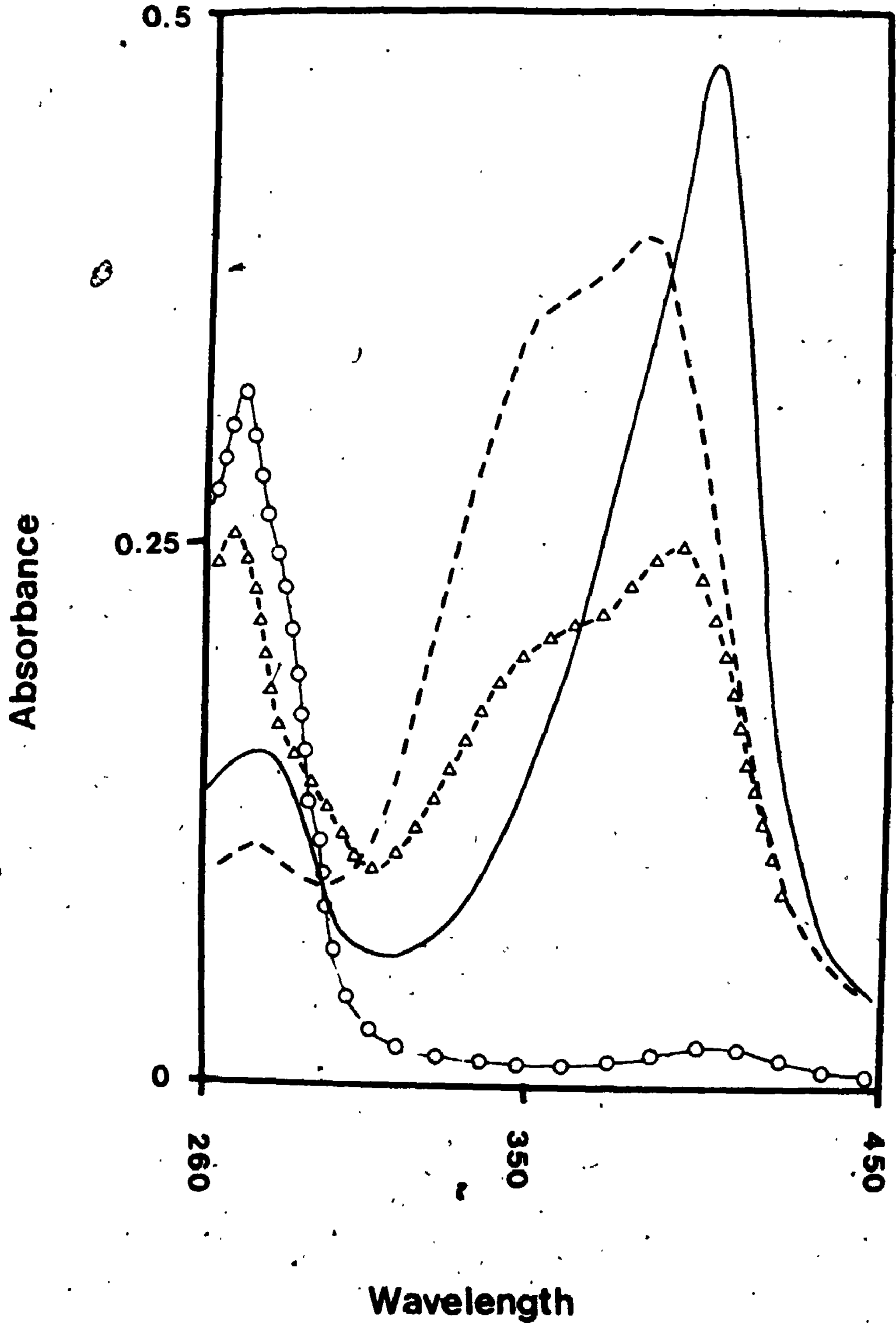
This evidence pointed to the fact that the cationic peroxidase was the major hemoprotein that was secreted by peanut cells in culture and hence found in the medium. This reinforced the earlier view expressed in Chapter 3, the spent medium of peanut cells in culture was a rich source of peroxidase. Therefore peroxidase could be purified in relatively few steps from the medium (Maldonado and van Huystee, 1980).

The validity of these results was supported by another observation in the same table (Table 12). In the supernatant of 177,000 g peroxidase formed only one fifth of the total hemoproteins labelled by ^{14}C ALA, since there were definitely other hemoproteins in the soluble phase of cell. Hence, this observation was not unexpected.

6.3.2 Cleavage of heme from the apoprotein by the cold acidified acetone method

The purified cationic peroxidase, holoenzyme, showed at least three fold more absorption at 407 nm than at 280 nm (Fig. 22). This meant a high RZ (>3.0) and indicated a satisfactory purity (Phelps and Antonini 1969). The apoprotein resulting from the cleavage of heme did not show an absorption at 407 nm, indicating complete removal of heme (Fig. 22). A similar conclusion was also derived from the fact that apoperoxidase (Fig. 21A, channel a) was stained only with coomassie blue (Fig. 21B),

Figure 22. Absorption spectra of peanut peroxidase (holo). (——); apoperoxidase (O O); hemin (········); and reconstituted peroxidase (Δ- -Δ).


0.5
0.25
0
Absorbance
260
350
450
Wavelength

while the holoenzyme stained in both cases for heme and protein (Fig. 21A and B, channel b). Table 13, also supported the separation from the heme moiety from apoprotein. Most of the radioactivity was associated with the heme, when the immunoprecipitated peroxidase from the medium and cytosol (177,000 g supernatant) was cleaved into heme and apoprotein. The residual amount of radioactivity that was still associated with the apoprotein might have represented the background.

6.3.3 Identification of the cleaved heme moiety

As a first step in identification, the cleaved heme moiety dried from ether was dissolved in alkaline pyridine and treated as described in section 6.2.3.1. It yielded a typical reduced minus oxidized pyridine hemochrome spectrum (Fig. 23), thus identifying the cleaved moiety as heme (Porra and Jones, 1963). This spectrum was also used to calculate the molar ratio of heme to apoprotein in cationic peroxidase. It yielded an equimolar ratio of heme to apoprotein. This was in agreement to the value given by Scandalios (1974) on the basis of homologies to other hemoproteins.

The isolated heme from peroxidase was chromatographed on reverse phase HPLC, for two reasons. One reason was to check if there was only one kind of heme moiety and secondly to tentatively identify heme moiety, based on retention times, given by Weinstein and Beale (1983). Recrystalized hemin was run as a standard for heme. The results presented in Fig. 24 showed that only a single peak could be detected, when either the isolated heme from peroxidase or hemin was chromatographed with the monitor set at 402 nm. This indicated probably

Table 13. Incorporation of ALA into the heme moiety of peanut peroxidase.

Fraction	Vol. ml	dpm x 10^3 Immunoprecipitate	Apoprotein	Heme
Medium	12	29.3	1.9	26.3
Supernatant 177,000 g	20	28.5	1.6	25.2

Immunoprecipitated peroxidase (section 6.2.7) corresponding to 1.0 ml of medium and of 177,000 g supernatant was cleaved into the heme and the apoprotein moiety, by the cold acidified acetone method in section 6.2.1. The heme in ether wasdried and the radioactivity measured in both heme and apoprotein. The data of 3 replicates was then calculated to give the results for total volumes.

Figure 23. Reduced minus oxidized pyridine hemochrome spectrum of the heme moiety extracted from the immunoprecipitate of the peroxidase.

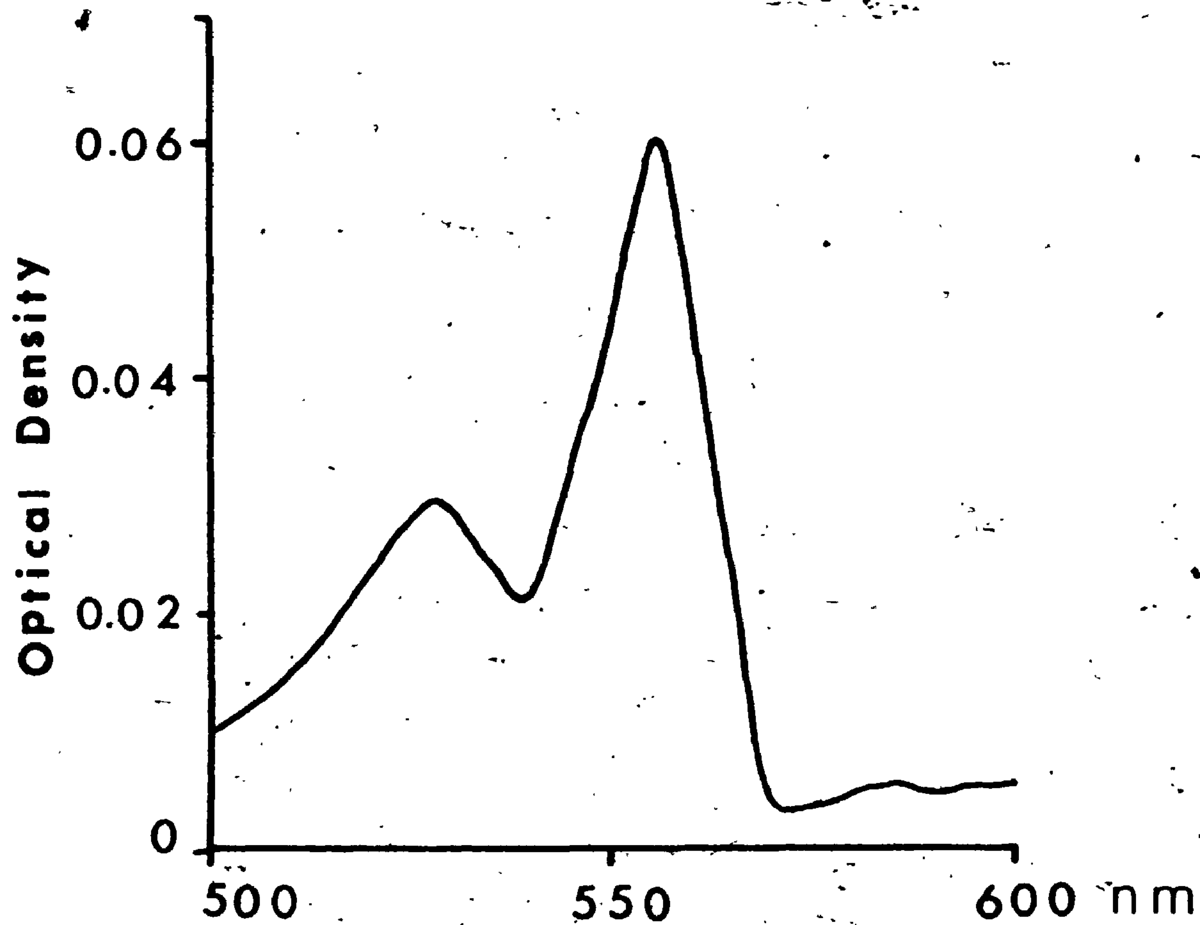
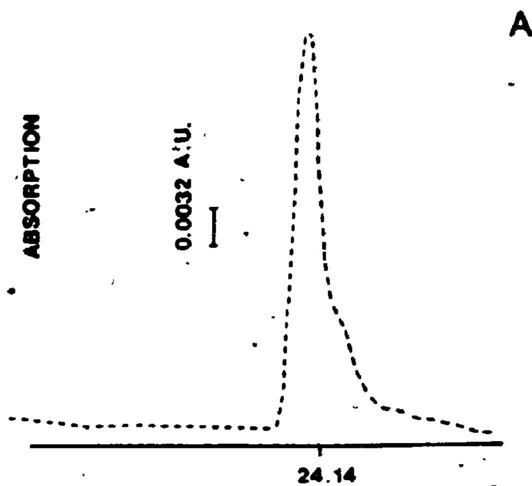
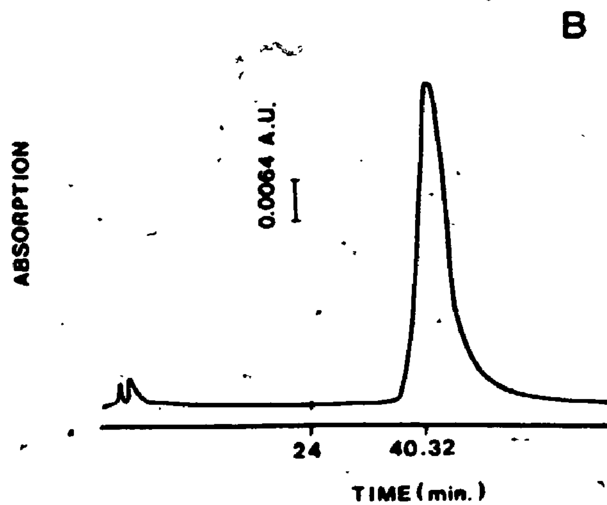
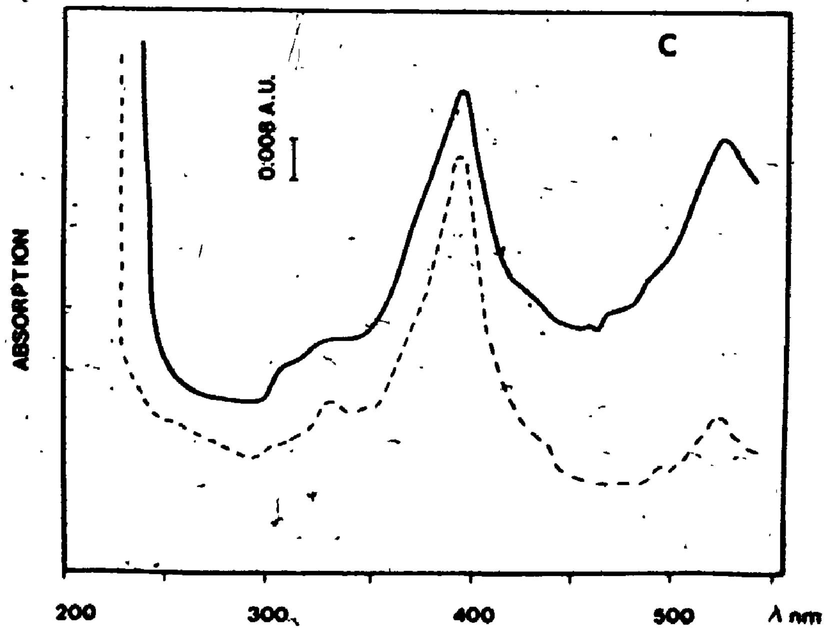

Optical Density
0.06
0.04
0.02
0
500
550
600 nm

Figure 24. HPLC separation of hemin (A) and heme extracted from peanut peroxidase (B). Conditions of run and other details were described in section 6.2.3.2.

Figure 24C. Absorption spectrum of hemin (.......) and heme extracted from peroxidase (———) detected on HPLC.


A
ABSORPTION
0.0032 A.U.
24.14


B
ABSORPTION
0.0064 A.U.
24
40.32
TIME (min.)


C
ABSORPTION
0.008 A.U.
200
300
400
500
λ nm

only one type of heme was present in peroxidase. The retention time of the heme from peroxidase (Fig. 24B) did not agree with that of hemin (this experiment Fig. 24A) nor protoheme nor heme 'a' published by Weinstein and Beale (1983). The HPLC run conditions were similar to those of Weinstein and Beale (1983) except the concentration of acetic acid in HPLC solvent was less than that used by Weinstein and Beale (1983). Nevertheless, both isolated heme from peroxidase and hemin gave identical absorption spectrum, thus suggesting that both were tetrapyrrole compounds.

The next step was to identify this heme by mass spectrometry, by identifying the major mass ion. The major mass ion, of the isolated heme from peroxidase, was found at 616 (Fig. 25), corresponding to the molecular weight of protoheme (Stecher _et al_., 1968). The other peaks with at least fifty percent intensity could be observed at mass ion 565 and 555 after the loss of a pyrrole ring or iron. Ion fragmentation pattern was not done, because once the major mass ion at 616 corresponding to protoheme was observed, the structure of protoheme is well established (Stecher _et al_., 1968).

6.3.4 Precursor(s) for heme moiety in peanut peroxidase

The results presented in Table 14 showed that most of the radioactivity associated with the heme moiety in peroxidase was derived from glutamic acid and not from glycine regardless whether the latter was labelled at C-1 or C-2 position. In the classical Shemin pathway of ALA synthesis (Fig. 26), following the condensation of glycine and

Figure 25. A portion of the mass-ion spectrum of the heme extracted from peanut peroxidase analysed on a MAT Varian 311A mass spectrometer. The mass spectrometer was set at 70 e.V. filter 100 Hz, with probe temperature at 350°C and resolution at 1000. The heme was dissolved in chloroform.

The figure showed that at the three sensitivities 10, 1 and 0.1 volts, the major mass ion peak corresponded to 616, the molecular weight of protoheme. On the 'y' axis is the intensity of the mass ions produced.

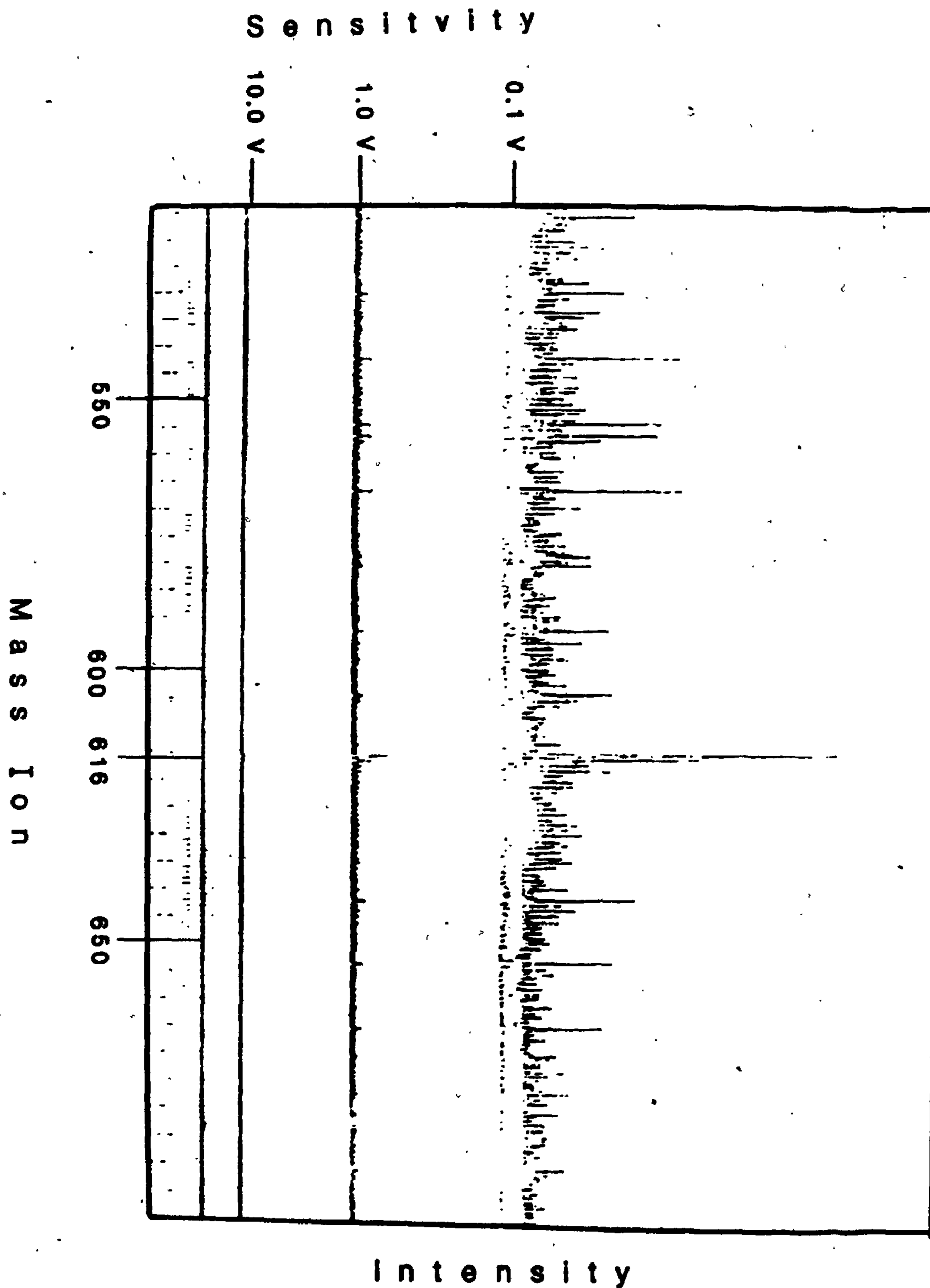
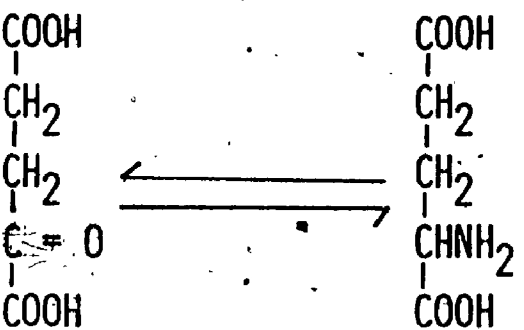

Sensitvity
10.0 V
1.0 V
0.1 V
550
600
616
650
Mass Ion
Intensity

Table 14. Incorporation of glycine and glutamic acid into the heme and apoprotein moiety of the peroxidase secreted into the medium by cultured peanut cells.

	cpm /2 ml of medium		Percent cpm in heme to that in apoprotein
Labelled amino acid	Apoprotein	Heme	
[1-^{14}C] Glycine (1.1 M Bq)	9,056	7	0.07
[2-^{14}C] Glycine (2.2 M Bq)	19,165	24	0.12
[U-^{14}C] Glutamic acid (1.1 M Bq)	4,868	255	5.20

The peroxidase immunoprecipitated from the medium (section 6.2.6), was cleaved by cold acidified acetone. The hemes in ether from the four replicates of each treatment were pooled together 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.

Figure 26. Schematic pathway of porphyrin synthesis (Granick and Beale, 1978). Animal and bacterial pathway is represented by broken line, plant pathway by solid line.



CH_2NH_2
$^*COOH$

COOH – CH_2 – CH_2 – C = O – S - CoA

CO_2

COOH – CH_2 – CH_2 – C = O – COOH

COOH – CH_2 – CH_2 – $CHNH_2$ – COOH

GLYCINE + SUCCINYL CoA → 5-ALA ← KETOGLUTARATE ⇌ GLUTAMATE

$^*CO_2$

ANIMALS + BACTERIA

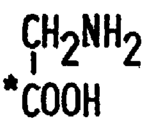
PLANTS

FE

HEMES ⇐ PROTOHEME ⇐ PROTOPORPHYRIN-9

MG

CHLOROPHYLLS



succinyl CoA to form ALA, the first carbon of the glycine is decarboxylated (Granick and Beale 1978, Akthar et al., 1976). Therefore, it was logical to use [1-^{14}C] glycine as control for [2-^{14}C]-glycine incorporation. Since no radioactivity could be measured in the heme fraction with glycine as precursor, it does not appear to be the precursor for heme in peanut peroxidase. It should be noted here that there was no obvious hindrance to the uptake of glycine by the cells as seen by the incorporation of glycine into the apoprotein moiety of immunoprecipitated protein as that (Table 14). Cells incubated with [U-^{14}C] glutamic acid showed, less of this amino acid as compared to glycine was incorporated into the apoprotein moiety. More of glutamic acid was detected in the heme moiety compared to glycine (Table 14). In addition, the free amino acid pool of glycine is half that of glutamic acid in these cultured cells (Verma and van Huystee, 1970). This together with the slightly higher molar ratio of glycine to glutamic acid in the peroxidase (van Huystee and Maldonaldo, 1982) explained the higher incorporation of glycine into the apoprotein than into the heme moiety. However, the higher incorporation of glutamic acid into the heme moiety, in spite of its relatively larger free pool in the cells, further strenghthened the results on the precursor of heme derived from this experiment (Chibbar and van Huystee, 1983b). This was consistant with the results of others which suggest that glutamate is the sole precursor for heme in red alga Cyanidium caldarium (Weinstein and Beale 1984).

Finally, it may be added that a 6 hour incubation period in this experiment and 4 hour incubation with ^{14}C-ALA in section 6.2.7 were used, because earlier studies had indicated the presence of a

considerable heme pool in peanut cells (van Huystee 1977b). The consideration that glutamic acid may be deaminated and transformed to succinyl CoA (Fig. 26) and as such may enter the porphyrin pathway, was rejected on the basis that [2-^{14}C] glycine was not incorporated (Table 14). Therefore succinyl Co-A would not be needed for heme biosynthesis.

6.3.5 Site of heme synthesis in cultured peanut cells

The elucidation of the site of heme synthesis in cultured peanut cells was necessary, since peanut cells cultured in a sucrose rich medium are achlorophyllous (van Huystee 1977c, van Huystee and Cairns 1980). It might be argued that heme destined for peroxidase, was a mere diversion of the porphyrin pathway in amyloplast (plastids) to heme synthesis (van Huystee and Turcon 1973). Thus, the site and precursor of heme may not represent the true picture in plant cells.

Peanut cells were incubated with ^{14}C-ALA for 4 hours (section 6.3.4) and the heme was extracted from amyloplast and mitochondria. Most of the radioactivity was associated with heme extracted from mitochondria (Table 15). This suggested that the mitochondria were the major site of heme synthesis in cultured peanut cells. Mitochondria do contain endogenous hemoproteins (eg. cytochromes) whose heme moiety might have been responsible for the radioactivity observed in this organelle. The slow turn over rate (1300 hours) of the cytochromes present in rat liver (Granick and Beale, 1978) rule out this possibility. Therefore, the data in Table 15 was taken as an indication of heme synthesis in the mitochondria as suggested by Granick and Beale (1978). These results also agreed with the earlier suggestion (van

Table 15. Site of heme synthesis in cultured peanut cells.

Organelle	dpm x 10^3 in heme derived from ^{14}C ALA	Total protein (μg)
Amyloplasts	2.3 ± 0.7	202
Mitochondria	33.2 ± 4.4	1,738

Amyloplasts and mitochondria were isolated from 15 to 20 g cells incubated with ^{14}C-ALA as described in section 6.2.7. Total heme from 3 replicates was extracted from these fractions as described in 6.2.1.

Huystee, 1977c) as well as with the electron micrographs which showed that the amyloplasts were laden with starch (Figure 17B). The methods to isolate the mitochondria and to check their homogenity were the same as reported in sections 5.2.1 and 5.2.3.

6.3.6 Site of attachment of heme to apoprotein

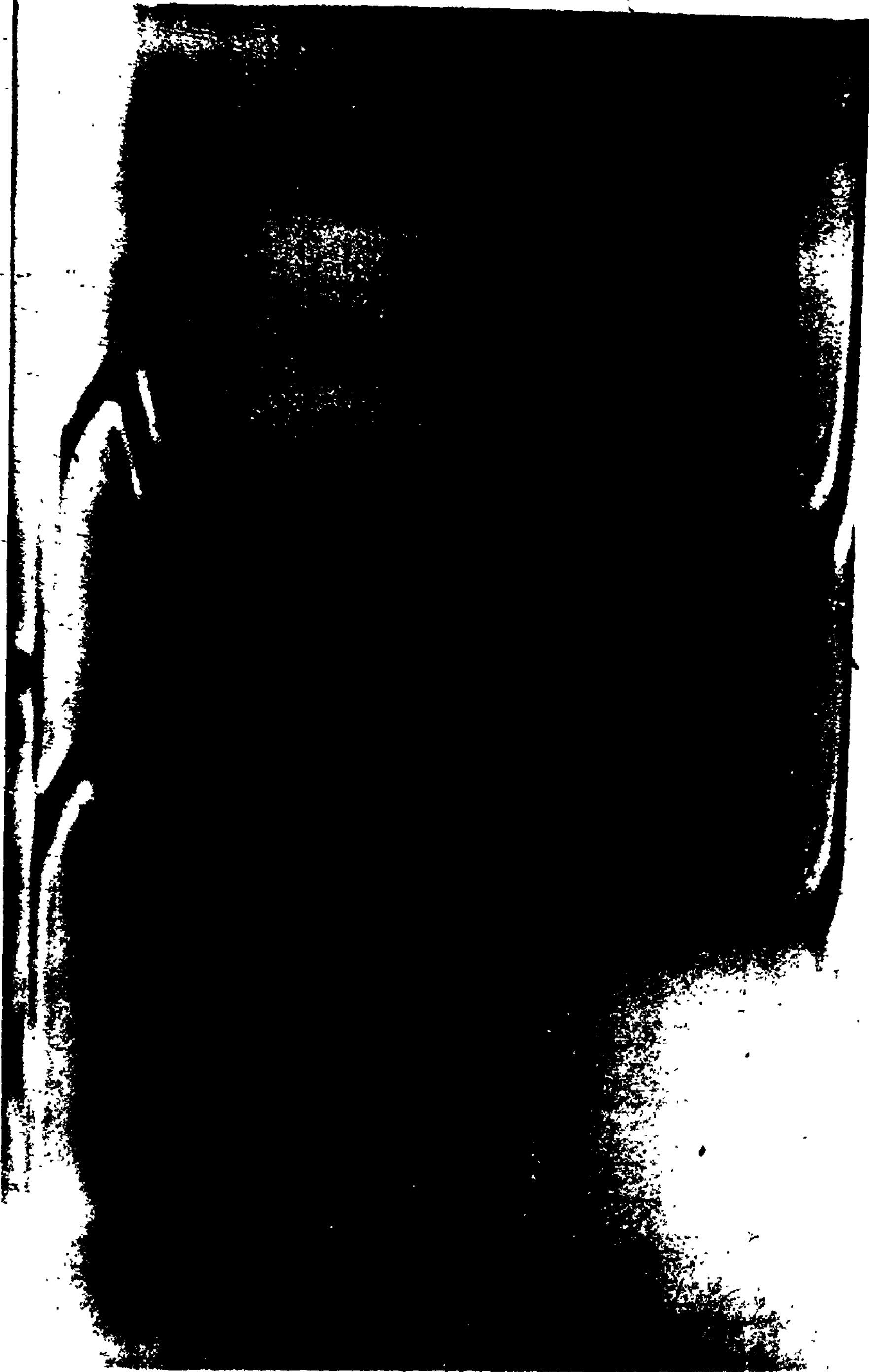
No conclusive evidence was available as to the precise site of attachment of heme to apoprotein during the overall biosynthesis of peroxidase. This awaits some future study on peptide sequencing. Nevertheless, the observation that the ^{14}C-ALA labelled peroxidase was detected in microsomes (Table 12) indicated heme was linked to the apoprotein, at that stage or prior to it.

6.3.7 Effect of removal of heme on the structure of apoprotein

The removal of heme from the holoenzyme does not significantly effect the migration of the peptide on SDS-PAGE (Figure 21B, channel a). This was expected considering the comparatively low molecular weight of heme.

The shape of the molecule as determined by analytical ultracentrifugation, did not appear to be drastically altered. The sedimentation coefficients (3S) for holoenzyme and apoperoxidase were found to be closely similar (Fig. 27). Similar results were also obtained with horseradish peroxidsae (HRP) isoenzymes of comparable purity (Phelps et al., 1971). However, these authors showed that at higher protein concentrations the sedimentation coefficients for the apoperoxidase is smaller than that of holoperoxidase, suggesting that the apoperoxidase has a higher frictional coefficient.

Figure 27. Ultracentrifugal analysis of the peanut peroxidase (holo) (2.2 mg/ml) in top row and the apoperoxidase (2.0 mg/ml) bottom row. Schlieren pattern at 64 minute of centrifugation. Sedimentation is towards right.



On the other hand, when the apoperoxidase and the holoenzyme were reacted with antibodies against the holo peroxidase in an Ouchterlony immunodiffusion assay, a precipitin arc was formed in both cases (Fig. 28). This suggested that the immuno-determinant sites were still intact and accessible to IgGs. This observation along with the results of sedimentation analysis of our data and earlier work (Phelps et al. 1971) suggested that the removal of heme did not appreciably alter the tertiary structure of apoperoxidase.

6.3.8 Enzymatic activities of apoperoxidase

In spite of the results reported in the preceding section, where no change in the tertiary structure of the apoperoxidase could be seen, the enzymatic activities associated with peroxidase, were significantly altered in the apoperoxidase. The peroxidative activity with all the substrates assayed was completely lost (Table 16). The IAA-oxidase activity was reduced at least twenty fold (Table 17). The loss of peroxidase activity agreed with the earlier reportes in literature (Siegel and Galston 1967, Srivastava and van Huystee 1973) since heme is a constituent of the active site (Ricard and Job, 1974). The decrease in IAA-oxidase activity by more than twenty times was in contrast to earlier reports (Siegel and Galston, 1967; Srivastava and van Huystee, 1973). In the determination of IAA-oxidase activity, we did not include any of the commonly used cofactors as Mn^{++} and DCP in the assay as in earlier studies. This could explain in part the differences observed in our studies, as well as others (Ku et al., 1970; Lee, 1977). But, it is now fairly well established that the true IAA-oxidase activity does not require the use of cofactors (Lee, 1977).

Figure 28. Ouchterlony immunodiffusion assay of the peanut peroxidase (holo) (10 μg) in wells (a) and (b) apoperoxidase (10 μg) in wells (c) and (d), against the antiserum raised with peanut peroxidase.

a
b
c
d

Table 16. Comparison of the peroxidase activity of the holo-enzyme and apoprotein of the cationic peanut peroxidase.

Substrate	Specific Activity (E.U./mg protein)	
	Holo-enzyme	Apoprotein
Guaiacol	637 ± 30	n.d.*
Eugenol	44 ± 6	n.d.
α-aminoantipyrine	62 ± 3	n.d.

* n.d. = not detected

± = S.E.

Table 17. IAA-oxidase activity of the holo, apo and reconstituted (apoperoxidase ± hemin) peanut peroxidase.

Enzyme	Specific Activity (µg IAA-oxidized/mg protein/min)
Holo-enzyme	12.3 ± 0.3
Apoprotein	0.5 ± 0.02
Reconstituted enzyme	5.0 ± 0.04

± = S.E.

6.3.9 Further evidence for the equimolar ratio of heme to apoperoxidase - Reconstitution experiments

Reconstitution experiments were performed to determine the molar ratio of heme to apoprotein in the peroxidase molecule, as well as to study the effect of heme on enzymatic activities associated with peroxidase. The reconstitution of the holoenzyme by the addition of heme to apoperoxidase was complete as determined by the absorption spectra at 280 and 407 nm of hemin alone and hemin reconstituted holoenzyme (Fig. 22).

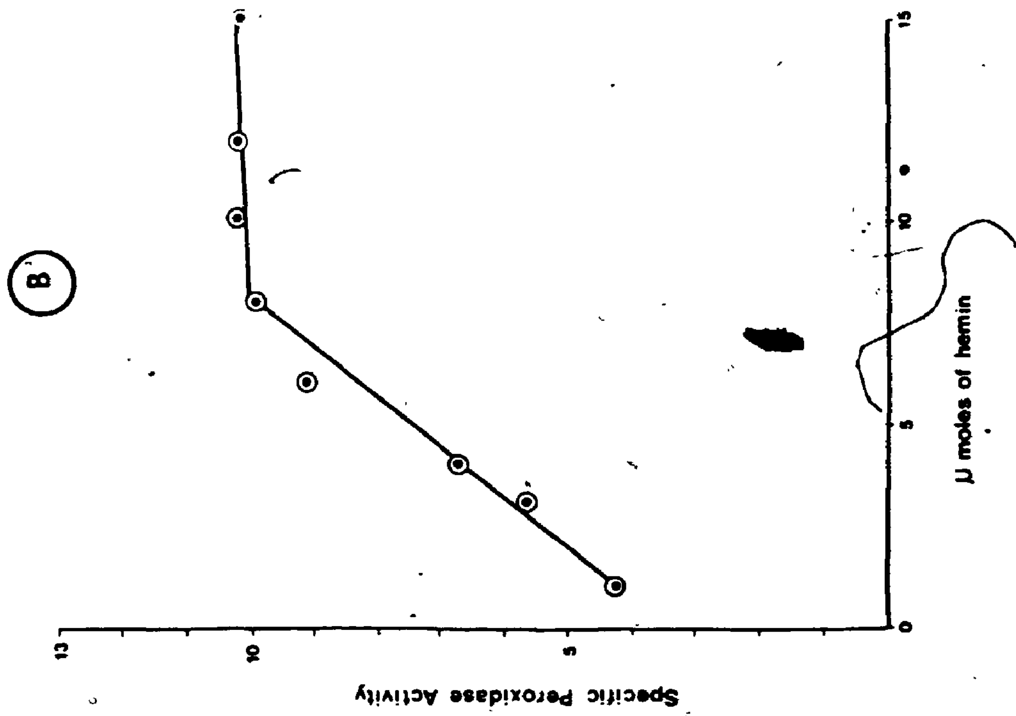
A change in the slope of the absorption at 407 nm of the apoperoxidase and hemin mixture occurred at 9 μm hemin (final concentration)(Fig. 29A). An experimental micromolar ratio of apoperoxidase to hemin of 10:9 is observed. This view point of an equimolar ratio is further reinforced by the data in Fig. 29B, where the peroxidase activity of the reconstituted enzyme was measured. The maximum activity was reached at a ratio of 10:8 of apoperoxidase to hemin. Thus both these pieces of evidence supported the data on measured contents of heme in peroxidase (section 6.3.3).

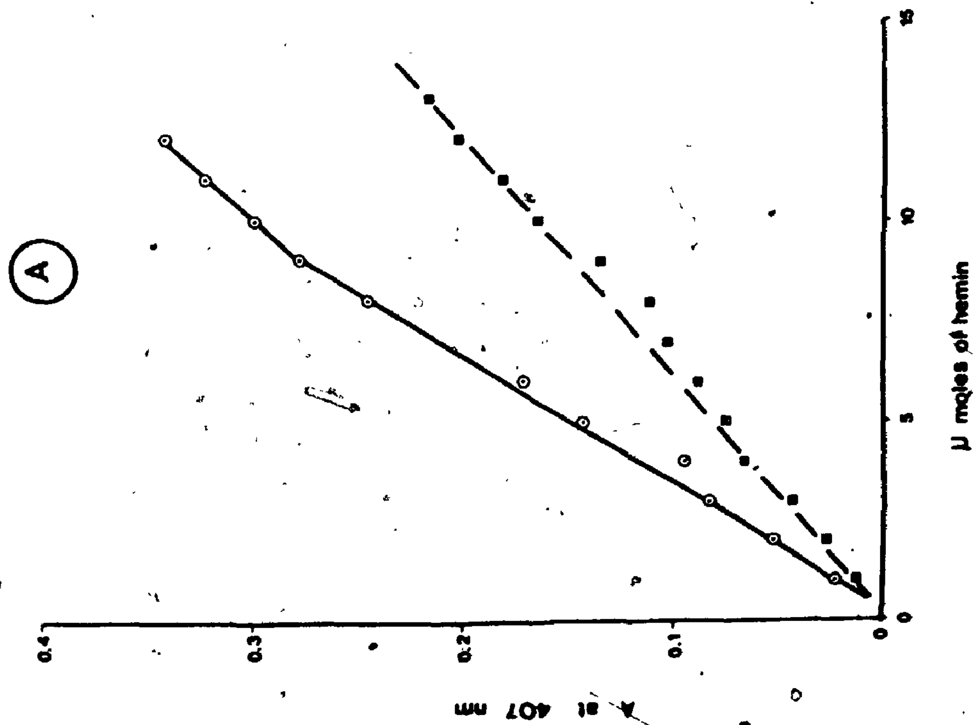
6.3.10 Enzymatic activities of the reconstituted peroxidase holoenzyme

The reconstituted peroxidase holoenzyme showed a ten-fold increase in the IAA-oxidase activity as compared to apoperoxidase alone (Table 17). The activity of the reconstituted holoenzyme was still approximately one half that of the native enzyme. Similarly the peroxidase activity of the reconstituted holoenzyme was almost one fifth that of the native enzyme. This may be due partly to the use of hemin, which forms hematin in alkaline solutions (Stecher et al., 1968).

Figure 29A. Reconstitution of the holo-enzyme with hemin and apoperoxidase. The apoperoxidase was kept at 10 μM and increasing amounts of hemin were added before measuring the absorption at 407 nm (⊙—⊙). The control was obtained by increasing concentrations of hemin alone (⊡—⊡).

Figure 29B. Specific peroxidase activity, using α-amino-antipyrine as substrate, of the reconstituted holo-enzyme formed by varying hemin concentrations. The concentration of apoenzyme was same as in 29A.


B
Specific Peroxidase Activity
μ moles of hemin


A
A at 407 nm
μ moles of hemin

Hematin has an additional hydroxyl group with iron, instead of iron (Fe^{++}) alone in protoheme. However, Ohlsson et al. (1977) reported that hematin or its derivatives are properly oriented in the prosthetic groups in HRP, based on circular dichroism studies.

6.3.11 Effect of heme deficiency in cultured peanut cells on the peroxidase secretion

Levulinic acid (LA) is an inhibitor of porphobilinogen synthesis (Beale 1970) thus blocking the synthesis of porphyrins and heme. It had been shown previously by van Huystee (1977a) that by incorporating 5 mM LA into the medium of cultured peanut cells culture ALA was found to accumulate in the cells indicating a block in the pathway. Therefore, by incorporating 3 mM LA in the medium of cultured peanut cells the release of peroxidase into the culture medium was delayed (Figure 30). The lag period was much smaller with 1 and 2 mM LA than with 3 mM LA. It was noted that 3 mM LA only induced a lag period and did not kill the cells, as the peroxidase activity increased in the medium after day 6. Further, this lag period could be reduced, if the cells were transferred to fresh medium without LA after day 3 (Fig. 30). Levulinic acid apparently did not have any effect on the measurement of peroxidase activity, as shown by assay of peroxidase in presence of LA. Peroxidase activity in the medium was shown to be proportional to peroxidase synthesis with the cells (van Huystee and Lobarzewski, 1982). Therefore, by circumstantial evidence it was suggested that heme may also control the synthesis of peroxidase as in the case of other hemoproteins (Hamilton et al., 1982). However, further work on the

Figure 30. Effect of levulinic acid on peroxidase released into the medium, by cultured peanut cells.

Peanut cells (8 ml settled cell volume in 50 ml final volume of cultured medium) were rinsed 3x with 3 volumes of fresh medium to remove adhered/residual peroxidase, before subculturing. Peroxidase activity was determined using guaiacol as substrate. (Representative of 3 experiments with similar results.)

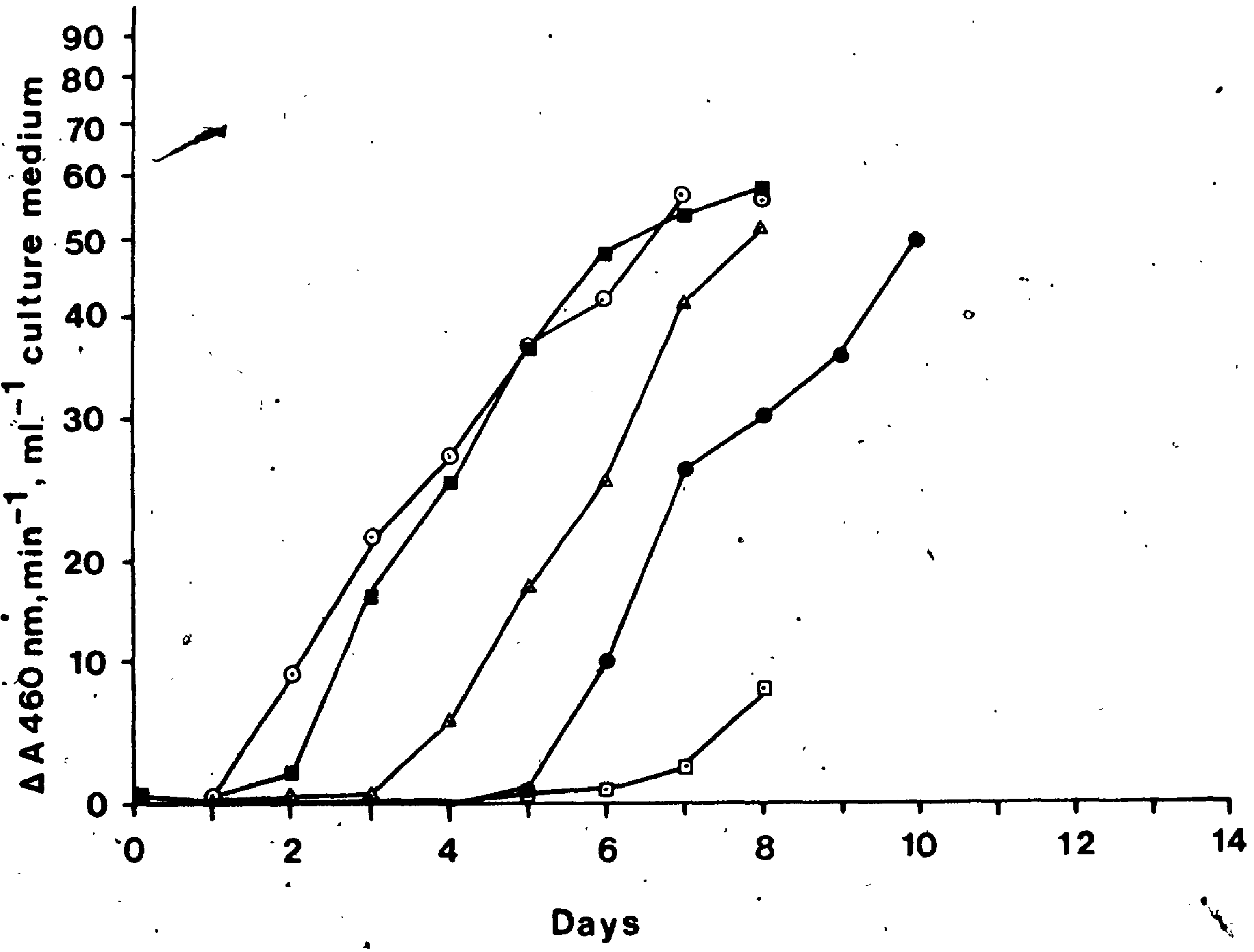
⊙——⊙ - control

■——■ - 1 mM LA

△——△ - 2 mM LA

⊡——⊡ - 3 mM LA

●——● - 3 mM LA removed after 3 days



ΔA 460 nm, min⁻¹, ml⁻¹ culture medium
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Days

in vivo and *in vitro* synthesis is needed to verify this preliminary result.

In summary heme in peanut peroxidase was identified as protoheme, which was present in an equimolar ratio to apoprotein. The protoheme was synthesized from glutamic acid in the mitochondria. Heme was involved in the various enzymatic activities associated with peroxidase. The results were suggestive that heme was linked to the apoprotein in or before the molecule reached the microsomal fraction. Heme might have an influence on the secretion of peroxidase by peanut cells in culture.

CHAPTER 7

FUNCTIONS OF PEROXIDASES IN PLANT CELLS

7.1 Introduction

The results reported in preceding chapters had reinforced the suggestion that cationic peroxidase was the major peroxidase isoenzyme secreted by peanut cells in culture (Maldonado and van Huystee 1980). This chapter examines (i) what possible functions it plays in peanut plant and (ii) the reason(s) for its secretion (Penel et al., 1984).

The studies on purified HRP preparation showed that the oxidative capabilities (using oxalacetate oxidation) of peroxidase are associated with the cationic peroxidases and the peroxidative activities with the anionic peroxidases (Kay et al. 1967). This view on supremacy of the cationic fraction in oxidation is further extended by circumstantial evidence based on enzymatic activities in crude cell/ plant extracts that the cationic peroxidases are associated with IAA-oxidase and the anionic peroxidases with lignin synthesis (Boyer et al. 1983 and references cited there in). However, this does not explain why the cationic form is secreted in peanut cells in suspension culture. In fact, the anionic form (involved in lignin synthesis) should be the major form that is secreted, because lignin synthesis takes place on the outer surface of cell. To check on the working hypothesis postulated above (Boyer et al. 1983), the anionic peroxidase (though in small quantities) was purified from the medium of cultured peanut cells.

These two peroxidase (anionic and cationic) were then compared as to their capabilities for different enzyme activities associated with peroxidase.

7.2. Materials and Methods

7.2.1 Isolation of the anionic fraction of peanut peroxidase

The anionic fraction was isolated from the "flow through" fractions of chromatography of crude medium proteins on a CMC column, as described in section 2.3.1. The anionic peroxidase forms only 10 to 15% of the total peroxidase activity in the medium (Maldonado and van Huystee 1980), thus the "flow through" fractions of 8 to 10 columns were pooled together. The proteins were precipitated with 80 percent ammonium sulphate. The pellet obtained after 10,000 g centrifugation was dissolved in 10 to 15 ml of 0.005 M Tris-Cl buffer at pH 8.4. This suspension of proteins was dialysed against two or three changes to a total of 4 to 6 litres of the same buffer over a period of 12 to 14 hours to remove ammonium sulphate. The dialysate was chromatographed over DEAE-sepharose in Tris-Cl buffer. The anionic peroxidase on this column was eluted with a gradient of 0 to 0.2 M NaCl in 0.005 M Tris-Cl buffer. The protein content of the effluent fractions was monitored on a ISCO UA2 monitor set at 280 nm. The RZ of the fractions were calculated as in section 2.3.1. Fractions with RZ values exceeding 2 were pooled. These were then concentrated by precipitating with 70 percent acetone and resolubilizing in 0.05 M phosphate buffer pH 7.0 for enzyme assays and physical determinations. Those fractions with RZ less than 2 were pooled, precipitated with 80 percent ammonium sulphate and

rechromatographed over DEAE-sepharose as described above. In order to obtain RZ of 2 the fractions were usually chromatographed twice over DEAE-sepharose.

7.2.2 Sedimentation analysis

The purified cationic and anionic proteins dissolved in 0.05 M phosphate buffer pH 7.0 were analysed in Beckman model E ultracentrifuge for sedimentation coefficient determination. The samples were centrifuged at 50,740 rpm at 20-25°C in an AN-E rotor and photographs with Schlieren optics were taken at 16 minute intervals. Sedimentation coefficients were calculated according to the method of Markham (1960) and corrected to water at 20°C.

The enzyme assays for peroxidase and IAA-oxidase were carried out as described in sections 2.4 and 2.5, respectively.

7.3 Results and Discussion

7.3.1 Homogenity of anionic peroxidase

The results presented in Figure 31 showed that a relatively homogenous anionic protein (channels a and f) and a cationic protein (channels d and e) had been isolated from the medium proteins. A double protein band (channel c) could be observed when the anionic and cationic proteins were electrophoresed together in one sample. This indicated a small variation in the molecular weight of the two proteins, as reported earlier (Stephan and van Huystee 1981). The cationic and anionic proteins exhibited RZ values of 3.6 and 2.0 respectively. This, was in contrast to the observations made for HRP, where higher RZ values were obtained for the anionic fraction than the cationic fraction

Figure 31. Comparison of molecular weight of anionic and cationic peroxidase from cultured peanut cells. Electrophoresis was carried out on a 5 to 17.5% gradient slab gel. Two concentrations for anionic (a, 10 μg and f, 20 μg) and for cationic (d, 40 μg and e, 25 μg) and 15 μg each for the combined sample (c) were used. Channel b carries marker proteins (serum albumin 67 kD; ovalbumin 43 kD; soybean trypsin inhibitor 20 kD and cytochrome (12.5 kD). Following electrophoresis the gel was stained with Coomassie blue.

a b c d e f

Figure 32. Ultracentrifugal analysis of the anionic peroxidase (0.7 mg/ml) alone (top row) and the anionic and cationic peroxidases together at a final concentration of 1.0 and 0.5 mg/ml, respectively (bottom row). Schlieren pattern at 32 minute of centrifugation. Sedimentation is towards right.

(Shannon et al. 1966). But the single protein band on SDS-PAGE (Figure 31) and a single peak in the ultracentrifugal analysis (Figure 32) showed that homogeneity of the anionic peroxidase was obtained. Cytochrome-c-peroxidase, which is also an acidic protein has a RZ of 1.25 (Yonetani, 1967). Thus, these variations on RZ values imply that RZ value alone based on heme absorption at 407 nm cannot be taken as a measure of purity for different isoenzymes. The RZ depends equally on the aromatic amino acid composition of each protein, which yields absorbance at 280 nm (Yonetani 1967).

7.3.2 Enzymatic activities of the anionic and cationic peroxidases

Summary of the results presented in Table 18 showed that no major differences in peroxidase activity between the anionic and cationic proteins could be observed. This was particularly true for the case of eugenol, which is considered to be a natural substrate for detecting the capability of lignin synthesis (Stafford 1960). Even for assays with guaiacol, a lignin precursor (Grand et al. 1979) the data were not greatly different considering the S.E. Furthermore, no differences were observed in the capability of each isoenzyme for IAA-oxidase activity (Table 18). Even the pH optima for the two proteins of IAA-oxidase activity was same (Berg et al., 1983). It was important to note that cofactors like Mn^{++}, DCP and H_2O_2 were not needed in IAA-oxidase assays for both proteins. However, the capability of the cationic peroxidase for IAA-oxidation was enhanced 4 to 5 fold with the addition of Mn^{++} and especially for DCP (data not shown). But the addition of cofactors and H_2O_2 does not really represent the true

Table 18. Comparison of the properties of the anionic and cationic fractions of peanut peroxidase.

	Peroxidase fraction	
	Anionic	Cationic
1. Molecular weight	42kD	40kD
2. Sedimentation coefficient	3.5S	3.5S
3. Specific peroxidase activity		
a) Guaiacol	907 ± 133	637 ± 30
b) Eugenol	37.0 ± 2.03	44.3 ± 6.4
c) α -aminoantripyrine	78.4 ± 0.005	62.5 ± 3.77
4. IAA-oxidase activity		
a) pH optima	3.6	3.6
b) specific activity (μg IAA oxidized, mg^{-1} protein.min^{-1})	10 ± 0.25	12 ± 0.35
5. RZ (A407/280 nm)	2.0	3.6

* ± S.E.

IAA-oxidase activity (Lee, 1977). Thus, the similarity of these two peroxidase fractions with opposite charges was in disagreement, with the general hypothesis put forward by Boyer et al. (1983) and as well as with the results of studies on HRP (Kay et al., 1967). Our results suggested that both proteins were potentially capable of performing the same enzymatic activities.

7.3.3 Variation in the nature of anionic and cationic peanut peroxidases

In spite of these similarities in catalytic properties of these two peanut peroxidases, they differ in their tryptic cleavage patterns (Stephan and van Huystee, 1981) and are not immunologically related (van Huystee and Maldonado, 1982). In addition, the cationic fraction accounts for three fourths of the peroxidase activity in the cell suspension medium (Maldonado and van Huystee 1980). The secretion of peroxidase into the extracellular fluid has been reported for studies on several plants as mentioned in Chapter 3. It was also shown that the peroxidase secreted by the cells in peanut leaf was immunologically related to the cationic fraction of peroxidase derived from cells (Chibbar and van Huystee, 1983a).

7.3.4 A hypothesis for the secretion of the cationic form of peroxidase

It has been noted in studies with rat brain, that the cationic form and not the anionic form of HRP is retrogradely transported in the soma of visual motor neurons (Bunt et al., 1976). These workers (Haschke and Friedhoff, 1978) later reported that the cationic form has 2.0 moles

of Ca^{++} per mole of protein as compared to 1.4 moles in the anionic form of horseradish peroxidase. The cationic form is also capable of exchanging the calcium, while the anionic is not (Haschke and Friedhoff, 1978). Calcium has been implicated in the structure of horseradish peroxidase by Wellinder (1979). The involvement of calcium in protein secretion in general is well known (Peterson and Maruyama 1984). Calcium stimulates the release of peroxidase into the medium of cultured beet cells (Sticher et al., 1981). This calcium stimulated peroxidase release is further enhanced by auxins in non-habituated, non-organogenic, cell lines in beet (Gaspar et al., 1983). However, auxins do not enhance this calcium induced secretion of peroxidase in habituated cell lines (Gaspar et al., 1983). Habituated cell lines do not require exogenous auxin for their growth. The necessity of calcium for auxin action is postulated (Roux and Slocum, 1982) and so is the necessity of a protein for auxin action (Went 1974). Auxin is transported from cell to cell (Goldsmith 1977). The transport of auxin is in the form of an anion (Hertel, 1983).

An auxin anion carrier protein has been implicated (Hertel, 1983), in auxin transport. Although, there is no definite evidence for the involvement of peroxidases, nevertheless it may function as an auxin anion carrier, considering the following circumstantial evidence:

(i) for auxin action, Ca^{++} is needed (Slocum and Roux 1982)

(ii) Ca^{++} forms an integral part of peroxidase structure

(iii) peroxidase is secreted and its secretion is Ca^{++} dependent

(iv) auxin travels in the form of an anion

(v) and the cationic form of peroxidase is capable of transport

In summary it seems quite plausible to involve cationic peroxidase in auxin transport. Cationic peroxidase has been recently implicated as an auxin receptor (Lobarzewski and Dawidowicz, 1983). Conversely there is no doubt that, evidence contrary to this has been presented namely that IAA-oxidase activity and auxin binding activities of maize membranes could be separated (Venis 1979). The main suggestion, intended to be made here, was that the cationic peroxidase appeared to have the potential to act as an auxin anion carrier and be involved in auxin transport and auxin action. The working hypothesis postulated by Bandurski (1982) that auxin is destroyed during the growth promoting act may be of interest here. More work on effect of Ca^{++} depletion, (using Ca^{++} ionophores) is required. Measurement of the level of IAA in the cells and in the medium, during the growth cycle of cells may lead to some conclusive results in future endeavours. The fact that the anionic peroxidase does not fulfill these criteria is of interest particularly since it appears to be an intracellular protein instead of extracellular one.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Peroxidase (E.C. 1.11.1.7) has been a favoured marker enzyme for altered plant growth and development (Scandalios and Soreson, 1977; Wetter and Dyck, 1983). The precise involvement of peroxidase in plant growth and development is not yet understood (van Huystee and Cairns, 1980, 1982). Generally an increase in peroxidase enzyme activity has been considered as an increase in peroxidase synthesis (Birecka and Miller, 1974; Taneja and Sachar, 1976), even though little is known about the biosynthesis of this hemoglycoprotein (Fig. 1). While HRP has been structurally examined, its biosynthesis in horseradish roots has not been investigated. Comparative studies on the structure of a few other plant peroxidases with HRP have revealed a diversity in structure of peroxidases (Mazza and Wellinder, 1980a,b; van Huystee and Maldonado, 1982). Therefore, indicating the necessity of the structural characterization and purification of peroxidases from the plant material in which it is to be studied.

A cationic fraction of peroxidase was isolated and characterized from the proteins secreted by peanut cells in suspension culture (Maldonado and van Huystee, 1980; van Huystee and Maldonado, 1982). The selective secretion of the cationic peroxidase was shown to be an energy dependent process (van Huystee and Lobarzewski, 1982). It was also shown that the secreted peroxidase originated from the peroxidase that

was extracted with a high salt buffer (van Huystee and Lobarzewski, 1982). Neither the intracellular origin of this secreted peroxidase nor the pathway of secretion of this peroxidase was identified.

In this study, the question of intracellular localization of peroxidase to explain the origin of high ionic extraction of peroxidase and its biosynthetic pathway was considered (Penel et al., 1984). The main feature of this study was the application of immunological techniques. The antibodies recognized the protein molecule (cationic peroxidase in this case) against which they were raised.

The first question to be considered was, whether the protein structure of the cationic peroxidase had changed during the transition of cells from explant to suspension culture (Chaleff, 1983; Meins, Jr. 1983). Immunodiffusion assays indicated that cationic peroxidase from cells in culture was immunologically related to that in the peanut plant and also to that which was secreted by the cells of the leaf. The significance of this relatedness was that antibodies raised against cationic peroxidase from cultured cells, could be used later in applied studies of the enzyme from peanut plants. It was shown by radioimmunassays, that the ratio of cationic peroxidase to total proteins was only one tenth in peanut leaves, as compared to that of peanut cells in culture. Secondly if molecular probes like cDNA, are developed later on, they may be used for growth and development studies on peanut plant (van Huystee and Cairns, 1982).

The studies by differential centrifugation coupled to radioimmunoassays on the intracellular localization of peroxidase during its biosynthesis were done. The results suggested that the peroxidase in

the high ionic extracts of cells originated from the microsomal fraction. This was in agreement, with the secretory nature of cationic peroxidase, and confirmed the earlier suggestion made by van Huystee (1978). In this study, monospecific IgGs were used for the first time for in vivo immunoprecipitation of peroxidase synthesizing polysomes. The results indicated a three to four fold higher peroxidase over protein synthesis in the membrane liberated- to free-polysomes. This strengthened the earlier suggestion made from in vitro studies on peroxidase synthesis by Stephan and van Huystee (1980, 1981). This technique of immunoprecipitation of peroxidase synthesizing polysomes, could be developed further to isolate the mRNA for peroxidase, as has been done for other low abundance proteins (Maurer, 1980; Shapiro and Young, 1981; Kraus and Rosenberg, 1982).

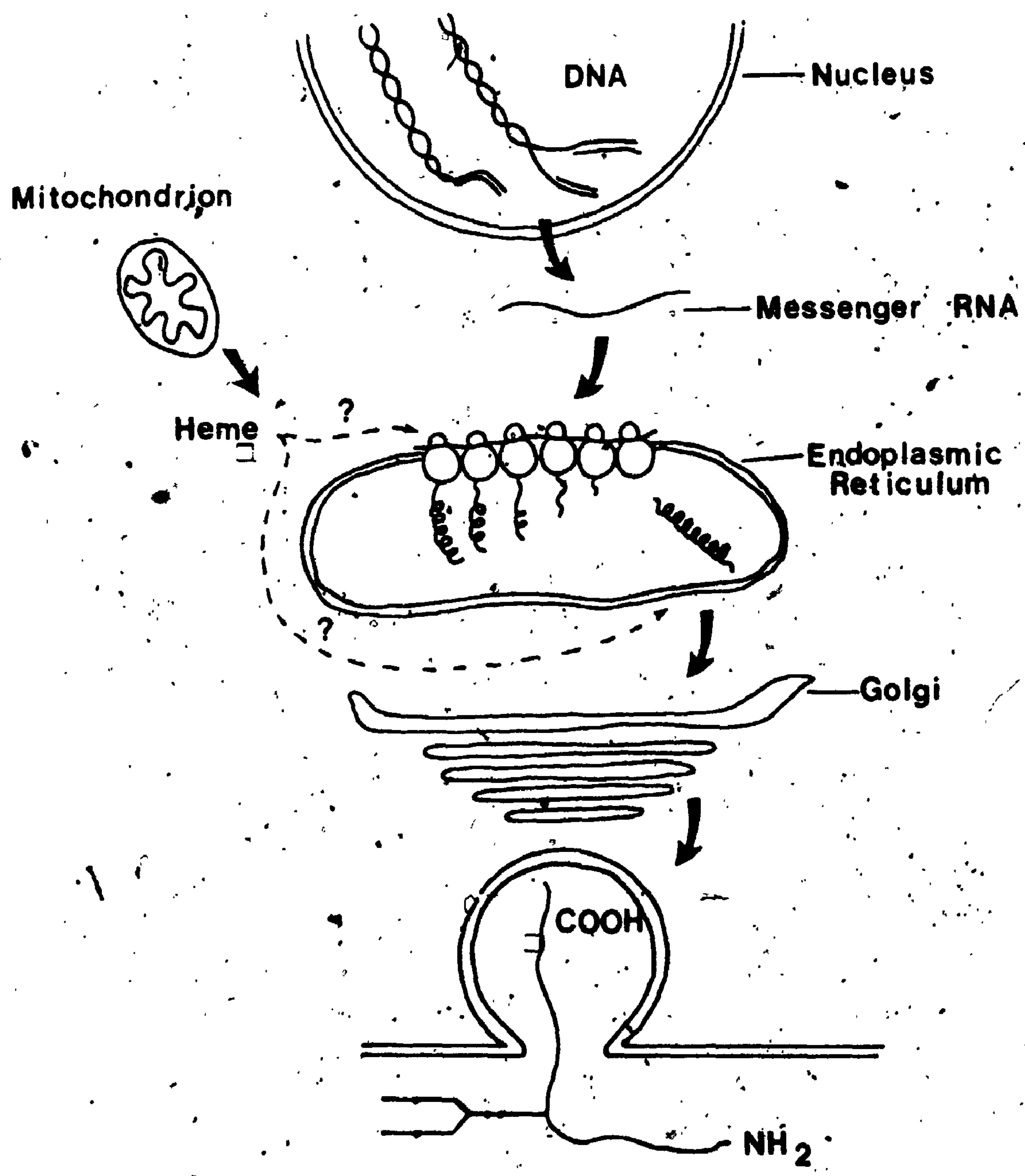
Heme moiety is an important component of peroxidase (Fig. 1). The precursor(s) for the biosynthesis of heme is(are) important, not only for peroxidase, but have been questioned since the discovery of glutamate as the precursor for ALA destined for chlorophyll (Beale and Castelfranco, 1974). Peanut cells in suspension culture, provided an ideal system, because of the achlorophyllous nature of cells in culture (van Huystee and Cairns, 1980) and the ease of isolation of hemoprotein by selectively immunoprecipitating the hemoprotein, peroxidase. Hence, it was shown that the heme moiety in peroxidase was derived from glutamic acid and not by condensation of glycine and succinyl CoA, as is the case in animals (Chibbar and van Huystee, 1983b). Weinstein and Beale (1984) subsequently reported that in red alga Cyanidium caldarum also, glutamate is the sole precursor for heme. It was also shown that

the heme moiety was synthesized in mitochondria. This was of interest, because arguments were raised, that since the cells do not contain chlorophyll, hence the synthesis of heme may be a mere diversion of the porphyrin biosynthetic pathway in plastids (amyloplasts) towards heme synthesis (van Huystee and Turcon, 1973). The heme moiety from peroxidase was identified as protoheme based on mass spectrometry. It was also shown to be present in equimolar concentrations to apoprotein as in cytochrome 'c' peroxidase (Yonetani, 1967). The peroxidase in the microsomal fraction had a heme moiety attached to it, as determined by radioimmunoassays.

Based on the observations, made in the current investigation and previous results from this laboratory (van Huystee and Cairns, 1982; van Huystee and Lobarzewski, 1982) a biosynthetic pathway for peroxidase and its release is proposed (Fig. 33). No other biosynthetic pathway for secretion of peroxidase has been proposed yet (Penel et al., 1984).

The current hypothesis for the action of peroxidases (Gaspar et al., 1982; Boyer et al., 1983) could not explain the secretion of cationic peroxidase in peanut cells in culture, as well as its involvement in plant growth and development (van Huystee and Cairns, 1982). The recent hypothesis (Boyer et al. 1983) suggested that cationic peroxidases are involved in IAA-oxidation, while anionic in lignin synthesis. This would imply that the anionic peroxidases should be secreted since lignin synthesis takes place on the outside of the cell membrane. However, evidence from different lines in our study (see section 7.3.4) indicated to the contrary. Moreover both the proteins (anionic and cationic) seemed to be capable of similar enzymatic catalysis,

Figure 33. A possible scheme for the biosynthetic pathway of cationic peroxidase in cultured peanut cells based on data from this study and previous preliminary studies with peanut cells.


DNA
Nucleus
Mitochondrion
Messenger RNA
Heme
?
Endoplasmic Reticulum
?
Golgi
COOH
NH2

thus disagreeing with the broad assignments of function proposed by Gaspar et al. (1982) and Boyer et al. (1983).

8.1 Prospects for Further Study

Many questions on the biosynthesis and function of peroxidases in plants have emerged from this study. The first major point of concern is the significance of the selective secretion of cationic peroxidase over the anionic peroxidases. Is the anionic form synthesized at lower levels as compared to cationic, so that it is secreted at lower rate? Conversely, the anionic form of peroxidase may be an intracellular enzyme and the cationic an extracellular enzyme. This compartmentation between the two forms of peroxidase may explain their role in plant growth and development (Chapter 7). The extracellular nature of cationic peroxidase, may be of interest to plant scientists in view of its use as a model for protein transport in animal motor neurons (Chan and Haschke, 1981). It has been reported (Bunt and Haschke, 1978) that the cationic isoenzyme 'c' of HRP is better capable of transport in the soma of motor neurons than the anionic form. The calcium related properties of the anionic and cationic forms of HRP are also of interest (Haschke and Friedhoff, 1978), since calcium has been involved in the hormonal action of plants (Roux and Slocum, 1982). However, for precise comparative studies, the first prerequisite is to purify the anionic peroxidase, as described in Chapter 7. Antibodies against this purified protein may be raised and used in determination of the biosynthetic rate of the anionic peroxidase, as reported in this thesis. Once the biosynthesis and structure of the anionic peroxidase is understood,

their role in growth and devleopment of peanut plant, may be investigated and compared to the cationic peroxidase, beyond the hypothesis made so far in this thesis.

Further clarification of the nature and origin of the cationic peroxidase from the low and high ionic extracts of cells is also needed. For this purpose, as well as to understand the regulation of its synthesis, a microsomal translation system may be developed (Miyata and Akazawa, 1983). Glycosylation inhibitors like tunicamycin, brassicin and compactin may be used to study their effects on peroxidase synthesis (Miyata and Akazawa, 1982).

The technique of the isolation of monospecific IgGs as well as the immunoprecipitation of peroxidase synthesizing polysomes, may be used to isolate the mRNA for cationic peroxidase. The mRNA may be used to develop cDNA probes for this peroxidase. These may then be used to investigate precisely the role of peroxidase in the various stages of growth and development (van Huystee and Cairns, 1982).

APPENDIX 1

Effect of monensin on the low and high ionic extraction of the peroxidase and proteins in cultured peanut cells.

Treatments	cpm x 10^3, mg^{-1} protein						Percentage peroxidase to total protein synthesis†	Ratio of high to low ionic extraction of peroxidase****
	TCA precipitated proteins			Immunoprecipitated peroxidase				
	Low*	High**	Total***	Low*	High**	Total***		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Control	91	66	157	0.9	2.7	3.6	2.3	3.0
Monensin 10^{-10} M	114	72.8	186.8	1.3	2.6	3.9	2.1	2.0
Monensin 10^{-8} M	78.6	82.8	161.4	0.8	3.6	4.4	2.7	4.5
Monensin 10^{-6} M	56.7	62.6	119.3	0.6	1.9	2.5	2.1	3.1

* Two gm F.W. cells incubated with 0.9 M Bq of ^{35}S methionine for 2 hours were treated for the last half hour with various concentrations of monensin. Then the cells were extraced in 0.05 M phosphate buffer pH 7.

** Subsequently the residue was re-extracted with the same buffer containing 0.08 M Kcl.

*** Proteins and peroxidase synthesized in the low and high ionic extracts taken together.

† Percentage of peroxidase to total protein synthesis

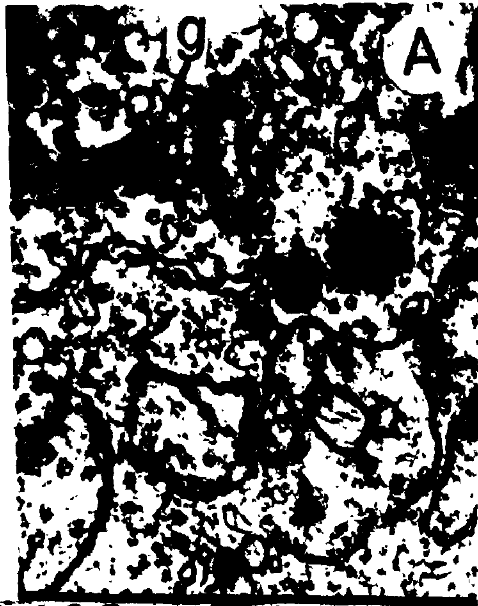
$$= \frac{\text{cpm for total peroxidase (column 7)}}{\text{cpm for total protein synthesis (column 4)}} \times 100.$$

**** Column 6/column 5.

Legend for Figure.

Peanut cells in culture (4 days old) were treated with 10^{-8} (Fig. B and C) and 10^{-6} (Fig. D and E) monensin for 30 minutes. Fig. A is control, not treated with monensin. Cells were prepared for electron micrography as described in section 5.2.4. g - represent Golgi apparatus. Magnification = 38,000x.

Monensin at a concentration of 10^{-6} M caused the disorganization of Golgi stacks.


g
A

B
g

C
g

D
g

E
g

APPENDIX - 2

Table 1. Distribution of RNA in free and membrane liberated polysomes during the period of active growth in cultured peanut cells.

Days of Culture	Fresh weight of cells (g)	RNA in polysomes (μg)			
		Free	Membrane liberated	Total	g^{-1} fresh weight
2	10	260	74	334	33.4
4	10	430	152	582	58.2
6	10	420	171	591	59.1
8	25	533	140	673	26.9

Cells were incubated with ^{35}S methionine (5 M Bq) for 2 hours. Polysomes (free and membrane-liberated) were isolated as described in section 5.2.2. Peroxidase synthesizing polysomes were immunoprecipitated as described in section 5.2.7. The radioactivity due to non-specific binding with non-specific IgGs was substracted from that obtained with specific IgGs. The net cpms obtained as a mean of 3 replicates from one experiment were shown in Tables 2 and 3. Protein synthesis was measured by the technique of Mans and Novelli (1961) described in section 2.9.2.

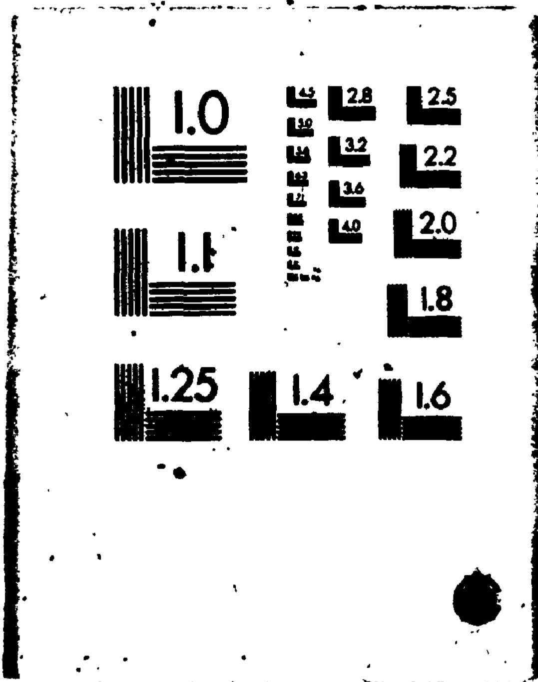

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

Table 2. *In vivo* peroxidase synthesis by polysomes during the period of active growth of cultured peanut cells.

Days of culture	Free	cpm in membrane liberated	Total	cpm x 10^3 mg^{-1} RNA	Ratio of membrane liberated to free
2	328	1,879	2,863	8.57	5.7
4	1,062	1,650	2,712	4.65	1.6
6	1,896	2,733	4,629	7.8	1.4
8	1,800	1,605	3,405	5.1	0.9

Table 3. *In vivo* protein synthesis by polysomes during the period of active growth of cultured peanut cells.

Days of culture	Free	10^3 x cpm in Membrane liberated	Total	cpm x 10^3 mg^{-1} RNA
2	27.4	11.4	38.8	116.4
4	29.4	11.9	41.3	70.9
6	39.3	15.7	55.1	93.2
8	33.2	10.2	43.4	64.5

Table 4. Percentage of peroxidase to protein synthesis in free-membrane liberated-polysomes and the two taken together.

Days of Culture	Percentage of peroxidase to protein synthesis*		
	Free	Membrane liberated	Total**
2	1.2	16.4	7.3
4	3.6	13.9	6.6
6	4.8	17.4	8.4
8	5.4	15.8	7.8

* Percentage of peroxidase to protein synthesis

$$= \frac{\text{cpm in immunoprecipitate}}{\text{cpm in TCA precipitate}} \times 100$$

** Total represents the membrane liberated- and free-polysomes taken together.

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