A COMPARISON OF THE FARNESYL PYROPHOSPHATE AND A-CYCLOPIAZONIC ACID SYNTHASES FROM *Penicillium cyclopium*

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Declaration

The work in this dissertation is my own unaided work and was done entirely at the University of the Witwatersrand, Johannesburg. No part of this work has been submitted in the past or is being submitted or is to be submitted for any other degree at any other university other than this degree.

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Duncan Harrison.

1st July, 1988.

ABSTRACT

Two enzymes of Penicillium cyclopium Westling, farnesyl pyrophosphate synthase [EC 2.5.1.1] and β-cyclopiazonic acid synthase, were purified. The pI of farnesyl pyrophosphate synthase was found to be 4.9 and the molecular weight was estimated as 77 600. β-cyclopiazonic acid synthase had a pI of 5.3 and an estimated molecular weight of 80 000. The specific yield of these enzymes per gram of mycelium was increased thirty percent by using aspartate rather than nitrate as the nitrogen source in the culture medium. Growth of Penicillium cyclopium on mycological peptone was found to completely suppress the production of α- and β-cyclopiazonic acid. The instability of the enzymes, found on the preparation of *mycelium by the ice shear press method* (McGrath et al, 1977), was circumvented by rupturing the mycelium through the preparation of either an acetone-dry ice or a freeze-dried powder. When the mycelium was ruptured by freeze drying rather than the preparation of an acetone-dried powder, the protein obtained from the aspartate-based medium was increased three fold and the specific activity of the crude mycelial preparation one and a half fold. EDTA and manganese, when present in the buffer used in the reconstitution of the freeze dried mycelium, were found to have destabilizing effects on the enzymes from the reconstituted mycelium. Glycerol on the other hand had a significant stabilizing influence on the enzymes from the reconstituted mycelium. Contrary to previous reports (McGrath et al, 1977) that the loss of enzyme activity on elution from an ion exchange resin was immediate and rapid, when freeze dried mycelium was used as the source of enzyme, both enzymes were found to be stable on elution with a linear gradient from a cation exchange resin. Sixty nine percent of the original enzyme activity remained five days after elution from the ion exchange resin.

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LIST OF ABBREVIATIONS

α-CPA	α-cyclopiazonic acid
β-CPA	β-cyclopiazonic acid
β-CPA synthase	β-cyclopiazonic acid synthase
cAAtrp	cycloacetoacetyl tryptophanyl
DEAE	diethylaminoethyl
DMAPP	dimethylallyl pyrophosphate
DTE	dithioerythritol
FPP	farnesyl pyrophosphate
FPP synthase	farnesyl pyrophosphate synthase (EC 2.5.1.1)
GPP	geranyl pyrophosphate
IPP	isopentenyl pyrophosphate
isomerase	isopentenyl pyrophosphate isomerase (EC 5.3.3.2)
β-MEtOH	2-mercaptoethanol
Me_2POPOP	1,4-bis-(4-methyl-5-phenyl-2-oxazolyl)-benzene
MW	molecular weight in daltons
PAGE	polyacrylamide gel electrophoresis
PPO	2,5 diphenyloxazole
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N',-tetramethylethylenediamine
tlc	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol

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CHAPTER ONE

INTRODUCTION

The existence of species of plants and microorganisms from which anomalous substances can be extracted has long been known to man. These anomalous substances, widely termed natural products, are produced by specific organisms in their natural habitats. Man has made use of them over the centuries for economic, medicinal and other purposes. Classical examples are rubber, caffeine and quinine. Man was however content to exploit these substances for his own benefit and was therefore only interested in ensuring an ample supply of the natural product and accordingly had not the slightest interest in what they were and how or why they were produced. As a result these substances have only been systematically studied during this century. The major thrust of the study arose from the discovery in the early 1960's that a natural product, aflatoxin, produced by Aspergillus flavus, was responsible for the death of a large number of turkeys that had eaten mouldy grain (Lancaster, 1961).

The discovery and characterisation of new natural products or secondary metabolites as they more correctly are known, has proceeded rapidly over the last thirty years. The focus of the study however has been the isolation and elucidation of the structures of secondary metabolites and their biosynthetic precursors and little attention has been given to the metabolic control of the biosynthesis of secondary metabolites - either the factors promoting or inhibiting their production, or the enzymes that are involved in these biosynthetic transformations. Encouragingly this state of affairs has begun to change over the last ten years.

In attempting to define the raison d'être of secondary metabolites, several theories have been advanced for the intended function of the production of secondary metabolites. An important earlier one was that secondary metabolites were produced in order to confer an ecological advantage on the producing organism (Mann, 1979). However once the multi-

farious character of secondary metabolites had been discovered, it became clear that an ecological advantage was conferred on only a small percentage of the species that produced secondary metabolites.

A more recent theory - one based on the metabolic significance of secondary metabolites rather than observations of their occurrence and effect in nature - was advanced by Foster (1947) and Woodruff (1966). This theory has since been developed and refined by Bu'Lock and Powell (1965) amongst others. Dealing specifically with fungi, Bu'Lock distinguished two phases in the growth of the organism. The first phase, the growth phase, he termed the trophophase and the second phase, where secondary metabolite production predominates, the idiophase. During trophophase growth is exponential and nutrients are in unlimited supply. Eventually however one or more essential nutrients becomes rate limiting and the growth rate is severely curtailed. A direct consequence of the reduced growth rate is that the utilisation of acetyl SCoA is reduced as well and therefore acetyl SCoA has the potential to accumulate (Bu'Lock, 1977). The accumulation of acetyl SCoA would lead to the unbalancing of metabolism and a concomitant loss of control. To avoid this the organism switches into idiophase and begins to produce secondary metabolites. Acetyl SCoA is diverted into the production of these secondary metabolites and thus the accumulation of acetyl SCoA is avoided and metabolic control is maintained. The transition from idiophase to trophophase is not a sudden step but occurs gradually in a stepwise manner as the enzymes required for the production of secondary metabolites are induced (Bu'Lock et al 1965).

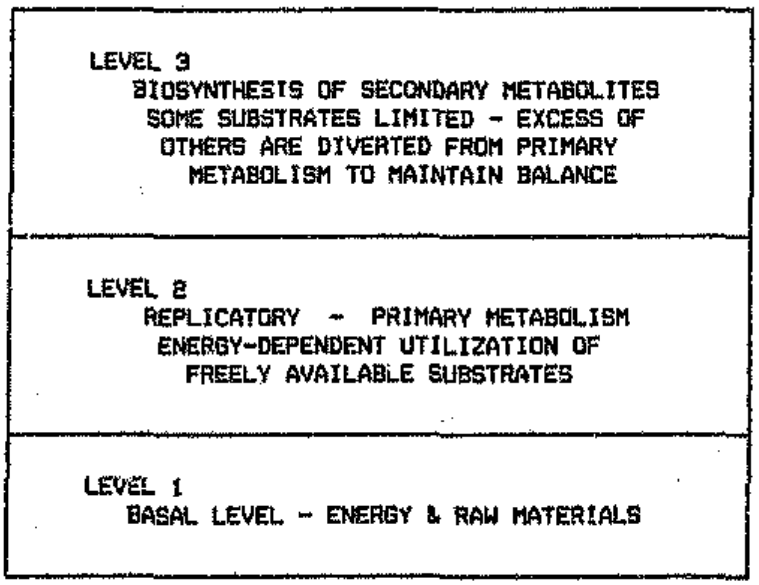
Martin (1977) clarified the issue by dividing the phases of fungal growth into three rather than two phases. These three phases are the balance, storage and maintenance phases. During the balance phase growth is exponential and the nutrients are in unlimited supply. When one or more of the nutrients become rate limiting the organism switches into the storage phase. The storage phase is characterised by reduced respiration, the accumulation of acetyl SCoA and the accumulation of fatty acids and carbohydrates. An example of the accumulation of lipids was reported by Neethling and McGrath (1977). They showed that the lipid concentration was

maximal on the second day of growth when the fungal metabolism was changing from trophophase to idiophase. The third phase of growth defined by Martin is the maintenance phase. In this phase the metabolic rate is minimal and all attention is devoted to maintaining balanced metabolism. One of the methods for achieving this is to divert excess metabolic intermediates into secondary metabolites.

Bu'Lock (1980) put the framework of this theory in perspective when he outlined three levels of metabolism (figure 1). These levels are firstly the basal level where energy and raw materials are provided, secondly the replication level where the energy-dependent utilisation of freely available substrates is harnessed to replicate the cell processes, and thirdly the secondary metabolite level where molecules of apparently little use are generated. The first two levels are tightly regulated and are almost identical across the broad spectrum of living organisms.

The third level, that producing secondary metabolites, is either the result of overproduction of replication level (level 2) metabolites such as sucrose or citric acid (Bhal 1986, Huttner 1987, Vining 1987), or the result of differentiation that results in the production of molecules that are unique in structure and are seldom found in more that three of four species (Bu'Lock 1977, Luckner 1983). Obviously the accumulation of acetyl SCoA can be avoided by either the biosynthesis of the unique molecules or the overproduction of replication metabolites. The overproduction of the latter begs the question as to whether the excess of these metabolites has any unbalancing effect on the metabolic control of the organism involved in overproduction and whether that organism has developed secondary controls to cope with this situation. The regulation of the third level appears on a macro scale to follow an inverse relationship with growth. The regulatory mechanism of the first two levels are well characterised in comparison to those of the third level where the diversity and singularity of secondary metabolites tends to limit their study. However it does appear that the biosynthesis of secondary metabolites is regulated in a similar manner to that of primary metabolism.

FIGURE 1 : Level of metabolism. The lower two levels are controlled by strict and specific mechanisms that are uniformly distributed over the whole spectrum of organisms (Bu'Lock, 1980). The rate of production of secondary metabolites is roughly inversely proportional to the rate of growth but the control mechanisms of secondary metabolism are little known or understood.



At a cellular level the induction of secondary metabolism has been ascribed to two processes. The first is the derepression of the enzymes involved in the bi ynthesis of secondary metabolites and the secon process is, where there is competition between a primary metabolic and a secondary metabolic substrate for a common substrate, the alteration of conditions to favour the production of the secondary metabolite. With secondary metabolites that are overproduced the control is likely to be predominantly through substrate competition or compartmentation. In the case of unique molecules that do not have any specific role but that are vital to the overall well-being of the organism, the induction of the enzymes responsible for the biosynthesis of these molecules is likely to be the controlling factor. Naturally it is possible that both these methods could act as dual controls on secondary metabolic pathways.

In summary, the basis of this theory of a balanced metabolic flux as *the raison d'Etre for secondary metabolism*, is that it is the metabolic process by which an excess of essential intermediates are removed from free circulation and thereby prevented from accumulating. Consequently the metabolic balance and control of the basal and essential replicatory processes of metabolism are maintained. The overall result is that the organism can keep its basic metabolic pathways operational and therefore survive albeit at a much reduced rate in the anticipation of circumstances favourable to renewed growth.

A typical example of secondary metabolite productio is that of α-cyclopiazonic acid by Penicillium cyclopium. The biosynthetic pathway is outlined in figure 2. Penicillium cyclopium is one of the penicillium and aspergillus sp from which so many secondary metabolites originate. Two other classic examples of the penicillium and aspergillus sp that spring to mind are Aspergillus flavus and Penicillium chrysogenum which produce the renown secondary metabolites, aflatoxin and penicillin respectively.

Dr B Scott in 1964 isolated several fungal species from samples of mouldy grain. One of these was the fungus Penicillium cyclopium. He showed that this fungus was responsible for the toxicosis found in animals that had eaten the mouldy grain. Holzapfel (1968) isolated the toxic principle

FIGURE 2 : An outline of the biosynthetic pathway of farnesyl pyrophosphate and α-cyclopiazonic acid.

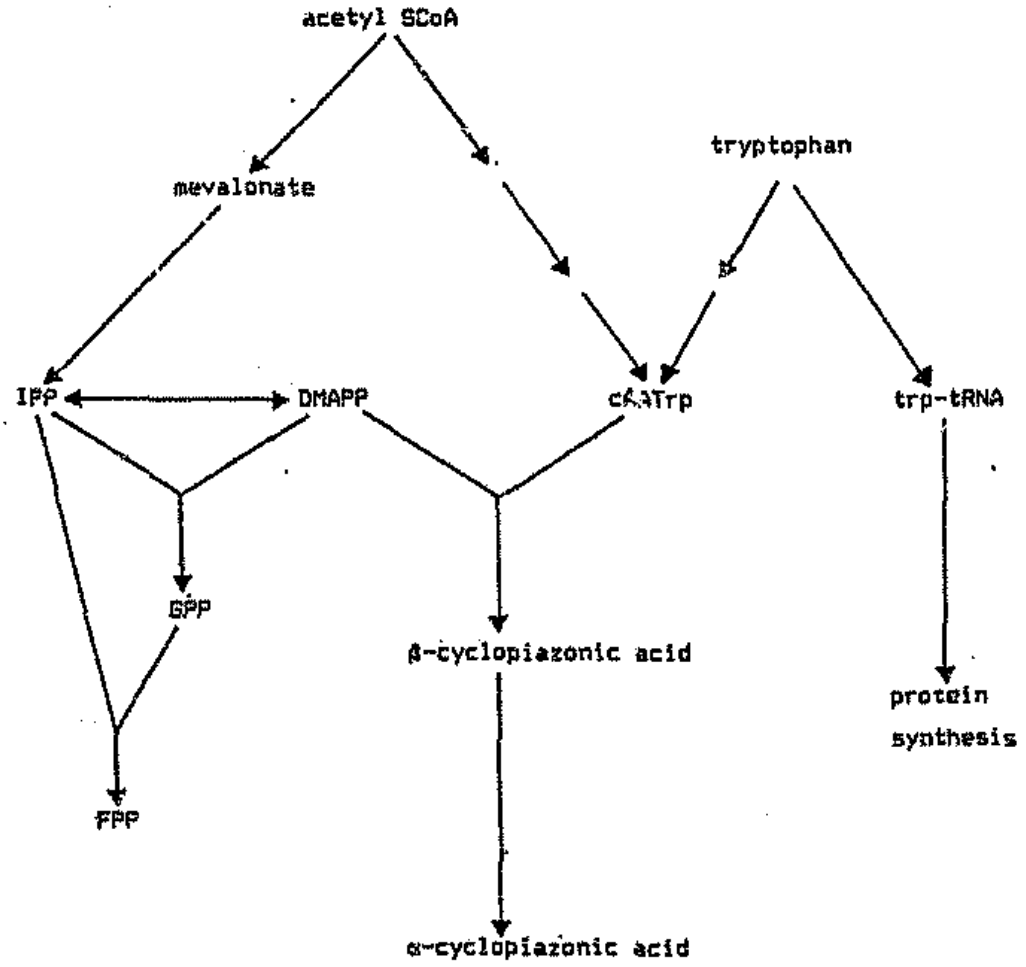
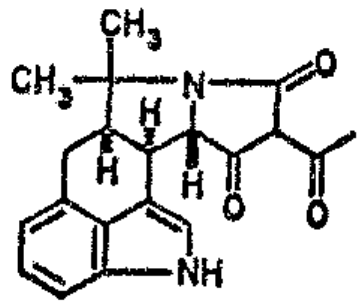
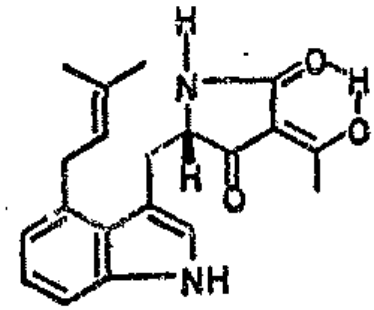


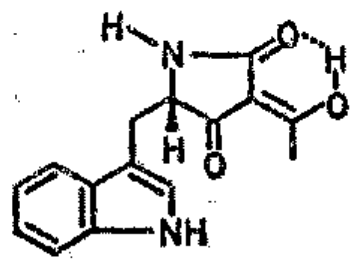
FIGURE 3 : Structures of some of the metabolites involved in the biosynthesis of α-cyclopiazonic acid by *Penicillium cyclopium*.



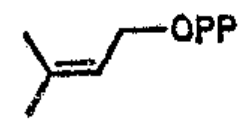
α-cyclopiazonic acid, I



β-cyclopiazonic acid, II



cycloacetoacetyl tryptophanyl, III



dimethylallyl pyrophosphate, IV

from this fungus and identified it as cyclopiazonic acid (I, figure 3). Holzapfel et al (1970) isolated bissecodehydrocyclopiazonic acid (β-cyclopiazonic acid - II, figure 3) from cultures of Penicillium cyclopium and Holzapfel and Wilkins (1971) showed that it was the immediate precursor of the cyclopiazonic acid. Cyclopiazonic acid (I) was renamed α-cyclopiazonic acid (α-CPA) to distinguish it from β-cyclopiazonic acid (β-CPA).

Holzapfel and Wilkins (1971) also reported that tryptophan (24.7%), mevalonate (7.0%) and acetate (3.5%) were incorporated in cyclopiazonic acid in varying amounts. They concluded that tryptophan was a biosynthetic precursor of α-CPA. The enzyme responsible for the conversion of β-CPA to α-CPA, β-oxidocyclase, was isolated and purified by Schabort et al (1971a, 1971b). The enzyme proved to be five isoenzymes and required a riboflavin derivative to effect the conversion. The reaction mechanism was studied extensively by Steenkamp et al (1973a, 1973b).

McGrath et al (1973), using cell free extracts prepared from cultures of Penicillium cyclopium, reported the isolation of another metabolite, α acetyl-3-(β-indoyl) methyltetramic acid (cycloacetoacetyl tryptophanyl - III, figure 3). They showed that it was a biosynthetic precursor of β-CPA. Later McGrath et al (1976, 1977) isolated and purified the enzyme from Penicillium cyclopium that converted cycloacetoacetyl tryptophanyl (cAAtrp) to β-CPA. The enzyme was designated dimethylallyl pyrophosphate: *cycloacetoacetyl tryptophanyl dimethylallyltransferase. In this report it* will be referred to as β-CPA synthase. They showed that β-CPA synthase adds the dimethylallyl moeity of dimethylallyl pyrophosphate (DMAPP - IV, figure 3) to the 4 position of cAAtrp. McGrath et al (1977) reported a molecular weight of 96000 daltons and a pI of 5.3 for β-CPA synthase. The Michaelis constants for β-CPA synthase were 2.0 μM for DMAPP and 6.0 μM for cAAtrp. They reported that β-CPA synthase had no apparent requirement for the divalent cations of Mg^{2+} or Mn^{2+} and that the presence of the latter had a disruptive effect on the enzyme activity of β-CPA synthase.

McGrath et al in 1977 reported the isolation of farnesylpyrophosphate synthase (FPP synthase - EC 2.5.1.1.) from Penicillium cyclopium. FPP

synthase has been shown to combine isopentenyl pyrophosphate (IPP) with dimethylallyl pyrophosphate (DMAPP) to form geranyl pyrophosphate (GPP) via a two step addition (Cornforth et al, 1966). It has also been shown to combine GPP with another IPP moeity to form farnesyl pyrophosphate (figure 2). Thus the FPP and β-CPA synthases compete for a common substrate, DMAPP, which is produced from IPP by isopentenyl pyrophosphate isomerase (isomerase - EC 5.3.3.2.). In their isolation of these three enzymes, McGrath et al (1977) reported a short study of the control mechanism which operated at this branch point between primary and secondary metabolism. The study in effect examined both the similarities between the competing enzymes and the metabolic basis for regulatory control. The study also elucidated several other factors that could influence the switch from primary to secondary metabolism.

There are several possible explanations as to how secondary metabolic enzymes arose. One possible hypothesis is that the enzymes involved in secondary metabolism such as β-CPA synthase are the result of a mutation in a primary metabolic enzyme that has resulted in an altered but functional enzyme (Schulz, 1978). This mutation must not be detrimental to the organism and may even give the organism a definite ecological advantage over its neighbors. If an enzyme of secondary metabolism was derived during the process of evolution from a mutated gene coding for a primary metabolic enzyme, one would expect such an enzyme to show a degree of homology with a corresponding enzyme of primary metabolism.

Such a similarity between a primary and a secondary metabolic enzyme would be evident under two different circumstances - these are firstly where their respective amino acid sequences have extensive homologies and secondly where the enzymes have identical subunits in common. Given that FPP synthase and β-CPA synthase compete for a common substrate and that the fungus, being eukaryotic, may well have multiple genes for the FPP synthase, it is conceivable that the β-CPA synthase could have arisen as a favourable mutation of one of these genes (Bajaj & Blundell, 1984). In order to establish whether it is possible for the two enzymes in question to have

originated from a common genetic precursor it would be essential to examine the subunit structure, the amino acid composition and the primary structure of both these enzymes to determine the degree of homology between them.

McGrath et al (1977) reported the molecular weights of these two enzymes as 64 000 and 96 000 respectively. The FPP synthase from various other sources has been shown to be composed of two identical subunits (Barnard and Popjak, 1981) and has a reported molecular weight in the region of 78 000 ± 2000 for the porcine liver FPP synthase (Yeh and Rilling, 1977, Barnard and Popjak, 1980), 74 000 ± 1400 for the human liver enzyme (Barnard and Popjak, 1981), 83 000 for the avian liver enzyme (Reed and Rilling, 1976) and 82 000 ± 2000 for the Saccharomyces cerevisiae enzyme (Eberhardt and Rilling, 1975). McGrath (personal communication) demonstrated that the two pertinent substrates (other than DMAPP), *IPP in the case of FPP synthase and cAAtrp in the case of β-CPA* synthase, can in certain conformations mimic a part of the structure of the each other. These overlapping parts are the keto groups on cAAtrp and the phosphate oxygens on the pyrophosphates of IPP. Thus there is the possibility that a small modification to the amino acid structure in the active site that would allow substrate substitution. In other words, were β-CPA synthase a mutation of FPP synthase, the catalytic site of β-CPA synthase could be marginally modified with cAAtrp becoming the preferred substrate. The binding coefficient of IPP to β-CPA synthase would be significantly less than that to FPP synthase. Furthermore, while IPP may bind to the active site of β-CPA synthase it cannot be transformed (McGrath et al, 1977).

It would be useful to compare β-CPA synthase with other fungal secondary metabolic enzymes to see whether there is a discernible pattern with respect to secondary metabolic enzymes which operate at branch points. Three relevant enzymes will be discussed below in this regard.

Takahashi et al (1978) reported the isolation of the enzyme DMAPP : aspulvinone dimethylallyltransferase from Aspergillus terreus. This enzyme catalyses the transfer of the dimethylallyl moiety of DMAPP to one of the

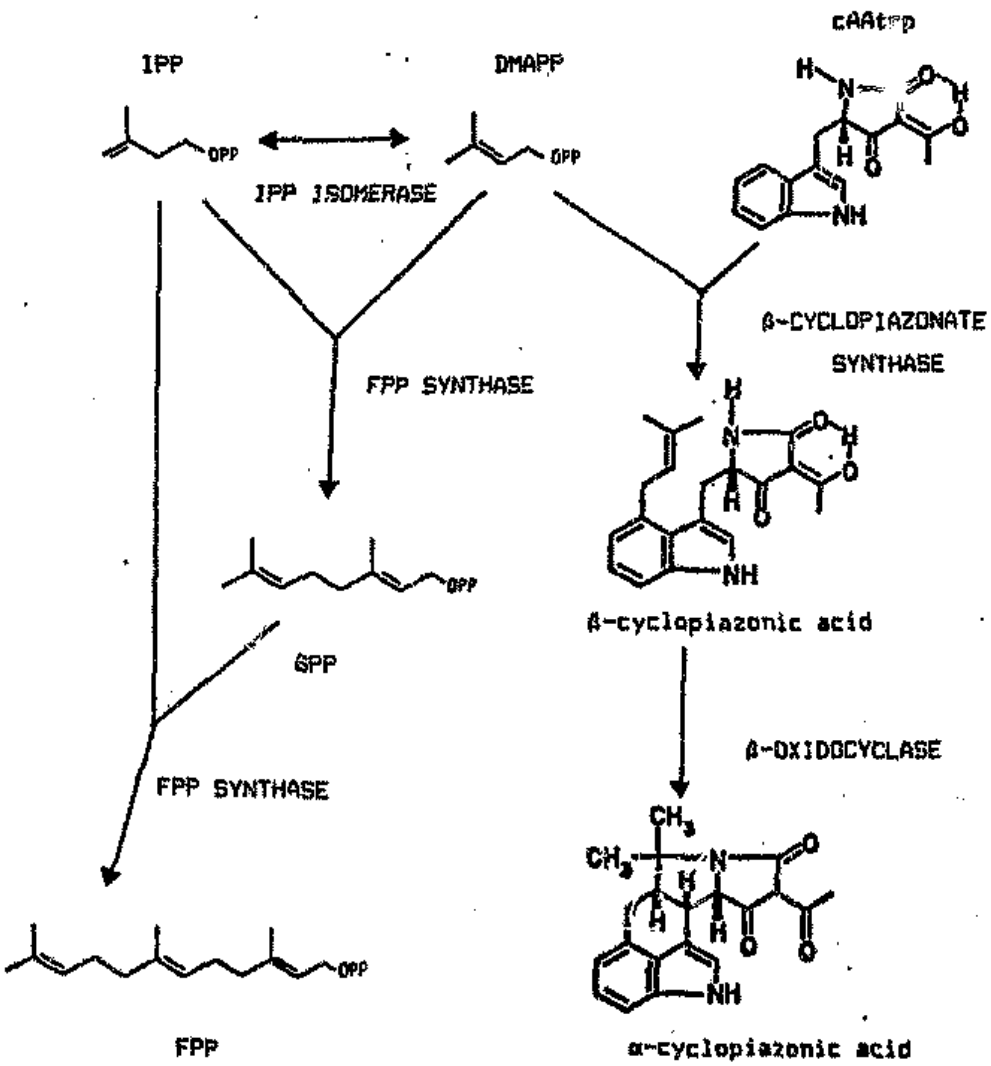
aromatic rings of aspulvinone E. The salient features of this enzyme are firstly that it has a similar subunit molecular weight (45 000) to the FPP synthase subunit (± 40 000), secondly that no metal ion is necessary for the activation of the enzyme and thirdly that sulfhydryl reductants have no effect on the functioning of the enzyme.

Heinstein et al (1971) and Lee et al (1976) reported the isolation and purification of DMAPP : L-tryptophan dimethylallyl transferase from Claviceps strain SD 58). This enzyme is the first one after the branch point in the synthesis of the ergot alkaloids. Cress, Chayet and Rilling (1981), studying the same enzyme, reported that in the absence of Ca^{2+} the enzyme is not inhibited by EDTA. They also reported that a divalent metal cation is not required for catalysis by this enzyme. While Lee et al reported that the enzyme was a monomer, Cress et al reported that it was dimeric with a subunit molecular weight of 34 000.

In both the above cases the secondary metabolic enzymes are dimeric prenyltransferases and are insensitive to chelating agents and are able to function in the absence of divalent metallic cofactors. McGrath et al (1977) reported similar properties for the secondary metabolic enzyme, β-CPA synthase.

The third secondary metabolic enzyme that will be discussed is produced by Penicillium ustus and species of Aspergillus amstelodami. The enzyme, dimethylallyl pyrophosphate : cyclo-L-alanyl-L-tryptophanyl dimethylallyl transferase (a prenyltransferase), catalyses the transfer of the dimethylallyl moiety from DMAPP onto cyclo-L-alanyl-L-tryptophanyl. The product formed is alanyl-2-(1,1-dimethylallyl)-L-tryptophanyl which is a biosynthetic precursor of secondary metabolite, echinulin (Allen 1972, Deyrup and Allen 1975). One of the results reported by Deyrup and Allen was the affinity of pig FPP synthase for a synthetic analogue, cyclopentylidineethyl pyrophosphate. This analogue shows a similar conformation to alanyl-2-(1,1-dimethylallyl)-L-tryptophanyl. The secondary metabolic prenyltransferase that forms alanyl-2-(1,1-dimethyl-allyl)-L-tryptophanyl however could not transform this substrate. This result is interesting in that the primary metabolic enzyme was able to

FIGURE 4 : Branch point of primary and secondary metabolism in Penicillium cyclopium.



transform an analogue of a secondary metabolite while the secondary metabolic enzyme was not.

The object of this research project was to purify and examine two enzymes of the fungus Penicillium cyclopium. These enzymes were the farnesylpyrophosphate synthase (EC 2.5.1.1.) and ß-cyclopiazonate synthase (McGrath et al, 1976, 1977) mentioned above. FPP synthase, the primary metabolic enzyme, is involved in the biosynthesis of squalene, while ß-CPA synthase, the secondary metabolic enzyme, is involved in the synthesis of the mycotoxin, α-cyclopiazonic acid (figure 4).

Initially the aim was to develop an improved isolation and purification procedure for the fungal enzymes, and having purified the enzymes to homogeneity, the homology of these two enzymes was to have been investigated. However the relative instability of the fungal enzymes led to the major part of this work being devoted to stabilising the enzymes and optimizing the steps in their purification procedure in order to be able to purify enough units of the enzymes for homology studies. McGrath et al (1977) reported that the enzymes were unstable and in a personal communication ascribed this instability to the use of the ice shear press to rupture the mycelium. The ice shear press places pressures of 500 psi and more on the enzymes and may have partially denatured them. The pressure may also account for the varying molecular weights that they reported. When this instability was found to persist even when other methods of mycelial rupture were used, a large part of this work was directed at stabilising the enzymes and establishing the purification protocol (with respect to time, resolution and the different characteristics of the protein that were used for separation). The criteria for the purification protocol were to firstly optimize both the yield of enzyme from a given volume of culture medium (i.e. to maximise initially the mass per 100 ml culture medium and then the enzyme yield per type of nitrogen source), and secondly to determine the most beneficial constituents for the reconstitution buffers used in the purification process. The FPP and ß-CPA synthases were then purified and their molecular weights estimated using gel filtration.

CHAPTER TWO

GENERAL METHODS

2.1 GENERAL

All chemicals used were of the analytical grade with the exception of the some of the trace elements in the culture medium.

2.1.1 Enzyme activity assays

One unit of enzyme is defined as the amount of enzyme that utilizes 1 µmole of substrate per minute at 30°C.

While both these enzymes are bisubstrate, only one substrate was used in determining enzyme activity. For farnesyl pyrophosphate synthase (FPP synthase) the substrate was isopentenyl pyrophosphate (IPP) and for β-cyclopiazonic acid synthase (β-CPA synthase) the substrate was dimethylallyl pyrophosphate (DMAPP). The FPP synthase utilizes two IPP molecules per molecule of FPP produced and therefore the actual results of the FPP synthase assay are twice the enzyme units of FPP synthase present and hence the enzyme units are halved to arrive at the correct number (figure 4).

The specific activity of an enzyme is defined as the number of enzyme units per milligram of protein.

The assays for the activity of the FPP synthase and β-CPA synthase as well as those for IPP-DMAPP isomerase were adapted from McGrath et al (1977). In the initial stages of purification the enzyme preparation still contained the isomerase which interfered with the assays of the FPP and β-CPA synthases. Under these circumstances a coupled assay was used since the veracity of the individual assays was doubtful. This coupled assay measured the combined effect of the isomerase and the synthases. The enzyme units derived from this assay are meaningful in relation to similar assays. Standardization of the assays was ensured by preparing and freezing aliquots of the buffer, divalent cations, the substrates and the reductant. In all assays the total assay volume, including the enzyme, was 1 ml. The constituents of the different assays were as follows.

COUPLED ASSAY : The buffer for the coupled assay contained 20 μmoles Tris maleate, pH 6.6, 1 μmole DTE, 5 μmoles magnesium sulphate, 39 nmoles cAAtrp and 50 nmoles [1-^{14}C]-IPP (50 000 dpm).

FPP SYNTHASE ASSAY : The buffer for FPP synthase contained 20 μmoles Tris maleate, pH 7.6, 5 μmoles magnesium sulphate, 1 μmole DTE, 50 nmoles DMAPP (unlabelled) and 50 nmoles [1-^{14}C] IPP (50 000 dpm). The results from this assay reflect twice the number of enzyme units since two [1-^{14}C] IPP molecules are combined into one FPP molecule.

β-CPA SYNTHASE ASSAY : The assay buffer for β-CPA synthase contained 20 μmoles Tris maleate, pH 6.4, 5 μmoles magnesium sulphate, 1 μmoles DTE, 78 nmoles cycloacetoacetyl tryptophanyl (cAAtrp) and 50 nmoles [1-^{14}C] IPP (50 000 dpm). 0.15 enzyme units of pig liver isomerase per assay were added and the mixture was incubated at 30°C for 30 minutes in order for the pig liver isomerase to reach equilibrium. The isomerase was inactivated by immersion in boiling water prior to the addition of the fungal enzyme. Sufficient DMAPP was provided in this manner to saturate the β-CPA synthase. Random sampling of the toluene layers from these assays was done to ensure that significantly more dpm were present in the toluene layer than were attributable to β-CPA or α-CPA alone since there had to be some DMAPP remaining at the end of the assay for it to be meaningful.

ISOMERASE ASSAY : The buffer that was used for the isomerase assay contained 20 μmoles Tris maleate, pH 6.0, 5 μmoles magnesium sulphate, 1 μmole DTE, 1 μmole manganese sulphate and 50 nmoles [1-^{14}C] IPP (50 000 dpm).

All assays were carried out at 30°C for 5 or 10 minutes. The assays were started with the addition of 100-200 μl of enzyme extract (0.1-1 mg/ml protein concentration) and stopped with the addition of 4 drops of $MgSO_4$-saturated HCl (conc) followed immediately by vortexing. For the FPP synthase and the isomerase assays 1 ml of Toluene solution (containing 1% of geraniol, farnesol, and nerolidol (v/v) each) was added to each assay tube and then mixed thoroughly. For the β-CPA synthase assay the 1 ml toluene added to the assay contained 72.9 nmol of α-CPA and β-CPA

(1.46 OD units, from uv estimation at 280 nm - log ε=4.32). For the coupled assay 0.5ml of each of these two toluene solutions was used. The suspension was broken by centrifugation (2000 rpm - 5 min.). From the toluene upper layer 200 µl were removed and added to 4.5 ml of a 2:1 toluene : Triton X100 scintillant (containing 34 mg PPO and 0.33 mg Me2POPOP). For the β-CPA synthase assay the 200 µl were removed and trickled onto a 2.5 cm disk of 3MM Whatman paper in the path of a stream of warm air from a hair dryer. The disks were then placed in vials containing the above scintillant. Counting was in an LKB Rediрac BetaRack scintillation counter for 10-20 minutes.

2.1.2 Protein determination

Protein was determined using the Bradford method. The method was adapted from Peterson (1983). On binding to the protein the absorbance wavelength of Coomassie blue dye shifts from 465 nm to 595 nm where the absorbance is read. The stock dye solution used for all protein determinations was made by dissolving 650 mg Sigma Brilliant Blue G in a 600 ml solution of 96% ethanol : 88% phosphoric acid (1:2). Immediately prior to use the stock dye solution was diluted with 3 parts of distilled water to form the dye reagent. Standards were made using Sigma BSA to the following concentrations - 5 µg/ml, 10 µg/ml and 20 µg/ml. The method of linear squares (see appendix) was used to construct a standard curve. Use was not made of a 50 µg/ml BSA standard since at concentrations higher than 20 µg/ml the calibration curve from the Bradford method loses its linearity. A reference sample of 20 µg/ml lysozyme was also prepared. If the reference's concentration was not within 20% percent of that of the 20 µg/ml BSA standard then the results were not acceptable and were repeated (Peterson, 1983).

Samples containing between 5 and 20 µg of protein were made up to 1 ml and aliquoted out. If the protein concentration of a sample was greater than 20 µg/ml it was diluted prior to the addition of the dye reagent. 1.5 ml Dye reagent was added to each sample and mixed. The samples

were allowed to stand for at least 5 but not more than 15 minutes and then read at 595 nm against distilled water on a Gilford 2400-S spectrophotometer.

2.1.3 Enzyme substrates

The substrate for the IPP-DMAPP isomerase and the FPP synthase assays, [1-^{14}C] isopentenyl pyrophosphate (IPP), was obtained from Amersham, U.K.. Labelled dimethylallyl pyrophosphate (DMAPP) was obtained using pig liver IPP-DMAPP isomerase that had been partially purified to free it of farnesyl pyrophosphate and phosphatase activity (2.2). Unlabelled IPP and DMAPP were prepared by R.M. McGrath.

The preparation of cycloacetoacetyl tryptophanyl (cAAtrp) was modified from that of McGrath et al (1976). Thin layer chromatography was used to monitor the synthesis. 500 mg of L-tryptophan were dissolved in 5 ml methanol and chilled to 0°C. 0.4 ml thionyl chloride were added and the mixture stirred for 16 hours. The mixture was warmed to room temperature and stirred for another 2 hours. In a biphasic ethyl acetate : ammonia - water system only a trace of ester was found in the aqueous layer while the reverse was found to be true for tryptophan. As a result methyl tryptophan was separated from the residual tryptophan using solvent : solvent extraction (ethyl acetate and 1N ammonia).

The ethyl acetate layer was evaporated to dryness in a stream of dry nitrogen. The residue, 508 mg of methyl tryptophan, was dissolved in 5 ml methanol and treated for 15 minutes with sodium methoxide (35 mg Na in 0.5 ml methanol). The mixture was cooled to -10°C and 0.5 ml Merck diketene added. The mixture was allowed to warm to room temperature and stirred for 20 hours. Once the reaction had reached completion the mixture was filtered and N-acetoacetyl tryptophan separated from methyl tryptophan on a Merck F_{254} 2 mm silica gel preparative tlc plate using a 20:5:3 (v/v/v) ethyl acetate : methanol : ammonia (conc) developing system. The N-acetoacetyl tryptophanyl was dissolved in benzene (20 ml) and cyclized by refluxing in sodium methoxide (40 mg Na in 0.6 ml methanol) for two

hours. The mixture was chilled to 0°C and extracted with distilled water. The benzene layer was removed and the water acidified with HCl (conc). Chloroform was added and 551 mg of cAAtrp extracted into the chloroform layer. The overall yield was 83.7%.

The synthesized cAAtrp was assayed in order to compare it with an authentic sample of cAAtrp (a gift from P.S. Steyn of CSIR). Equal amounts of the synthesized cAAtrp and authentic sample were made up in the assay buffer. The results of the β-CPA synthase assay showed that the synthesized product was 21% more active than the authentic sample.

2.2 PURIFICATION OF PIG LIVER ISOMERASE

2.2.1 Purification

All procedures were carried out at 4°C. A pig's liver was obtained from the abattoir and frozen immediately. The liver was allowed to thaw until it was only semifrozen and 100g was diced. The diced liver was homogenized for a minute in one and a half volumes of extraction buffer (20 mM Tris maleate, pH 6.4, 1 mM DTE and 1 mM EDTA) using an Ultra Turrex homogenizer. The homogenate was centrifuged at 16 300g for 20 minutes and the pellet discarded. The supernatant was centrifuged at 48 000g for 3 hours and the pellet discarded. The supernatant was filtered through Whatman No 4 filter paper (to remove lipids) directly into Visking 9-36/32' dialysis tubing. The filtrate was concentrated against sucrose for 4-5 hours until the supernatant volume was less than 15% of its initial volume. The dialysis tubing was tightened and the filtrate dialysed with stirring for 16 hours against a litre of dialysis buffer (20 mM Tris maleate, pH 6.4, 1 mM DTE). The dialysis buffer was changed twice. After aliquots had been withdrawn for protein and enzyme activity determination, the concentrate, together with Dextran Blue, was loaded onto a 80 cm x 2.6 cm Ultrogel AcA 44 gel filtration column (fractionation range - 10 000 - 130 000 daltons), which had previously been equilibrated for 24 hours with buffer (10 mM Tris HCl, pH 7.5, 1 mM DTE). 400 mg of

protein (14½ ml) were loaded onto the column. The flow rate of the column was 18 ml/hour. Fractions of 13 ml were collected and assayed for protein and enzyme activity. Haemoglobin was used as an indicator of the isomerase's position in the eluent as the isomerase eluted immediately after it.

2.2.2 Ion Exchange

DEAE cellulose (Whatman DE 52) was the cation exchange resin used. 250 ml of DE 52 per run was used. The resin was prepared by stirring the gel resin in 0.5 M HCl for 2 hours. This was followed by stirring in 0.5 M NaOH for another two hours. After the ion exchange resin's pH had been adjusted to 7.0 with HCl, it was washed in 3 volumes of 100 mM Tris HCl, pH 7.0. The resin was poured into a 20 cm x 4 cm column and equilibrated with 6 volumes of 20 mM Tris HCl, pH 7.0. The active isomerase fractions *from the AcA 44 gel filtration column (2.2.1) were pooled and* loaded onto the ion exchange column. Any isomerase fractions where contamination by FPP synthase was found or even suspected were excluded. The column was then washed with another 3 volumes of the same buffer. The absorbance of the wash was monitored using a LKB Uvicord 4701A at 254 nm and the washing of the column continued until the absorbance had returned to that of the baseline prior to loading. The wash was assayed for the presence of enzyme activity but none was found. The enzymes were eluted using a linear gradient (100 mM-400 mM Tris HCl, pH 7.0, 1 mM DTE). Fractions of 7 ml were collected and assayed for protein and enzyme activity. The protein remaining on the ion exchange resin was eluted with 400 mM KCl.

2.2.3 Storage

Since the isomerase was only required for the synthesis of DMAPP, once it was essentially free of FPP synthase, 500 µl fractions with a specific activity of 0.21 enzyme units/mg and a protein concentration of 1.44 mg/ml were aliquoted into eppendorf tubes and stored in liquid nitrogen where they were stable for up to 9 months.

2.3 THE ORGANISM - *Penicillium cyclopium*

2.3.1 Standard Growth

Penicillium cyclopium Westling ATCC 7615 (formerly CSIR 1082), the fungus used, was obtained originally from the CSIR. In these experiments the primary innoculum was started using freeze-dried slants prepared by R.M. McGrath. The slant was flooded with sterile culture medium and allowed to soak for 15 minutes, after which it was homogenized with an Ultra Turrex homogenizer. The standard culture medium used for the growth of the fungus was a modified Czapek culture medium developed by Neethling (1972). The constituents are shown in table 1. All culturing procedures were carried out under sterile conditions and growths were repeated if the growths differed noticeably from each other. The culture medium was made in bulk and 100 ml aliquots were dispensed into 500 ml erlenmeyer culture flasks. The flasks were autoclaved at 121°C for 15 minutes before use. Both the primary and secondary innocula were grown in a constant temperature room (25°C) at 180 rpm on a New Brunswick G10 gyrotory 5 cm orbital shaker.

All cultures were started with a three day growth of the primary innoculum using the standard culture medium. After the primary innoculum had grown for 3 days it was homogenised using an Ultra Turrex and 5 ml aliquots transferred to new flasks to form the secondary innoculum. The secondary innoculum was grown for between two and five days depending on the nature of the experiment. If any of the growths of the secondary innoculum clumped after the initial 24 hours of growth they were homogenised with the Ultra Turrex until the lumps had been broken up. Growth was stopped by separating the mycelium and the culture medium by filtration. The mycelium was frozen and the culture filtrate stored at 4°C with a trace of chloroform if not immediately used.

TABLE 1 : The nutrients of the standard culture medium, based on those of Neethling (1972), are shown in the table.

STANDARD CULTURE MEDIUM CONSTITUENTS	
Glucose	60 g
Sodium nitrate	4.5 g
Dipotassium hydrogen phosphate	121 mg
Potassium dihydrogen phosphate	879 mg
Magnesium sulphate	500 mg
Potassium chloride	500 mg
Sodium borate	7.0 mg
Ammonium molybdate	5.0 mg
Copper sulphate	3.0 mg
Manganese sulphate	0.11 mg
Ferrous sulphate	10.0 mg
Zinc sulphate	17.6 mg
pH adjusted to 5.5 and made to	1000 ml
post sterilization pH	5.8-5.9

2.3.2 Rupture of the Mycelium

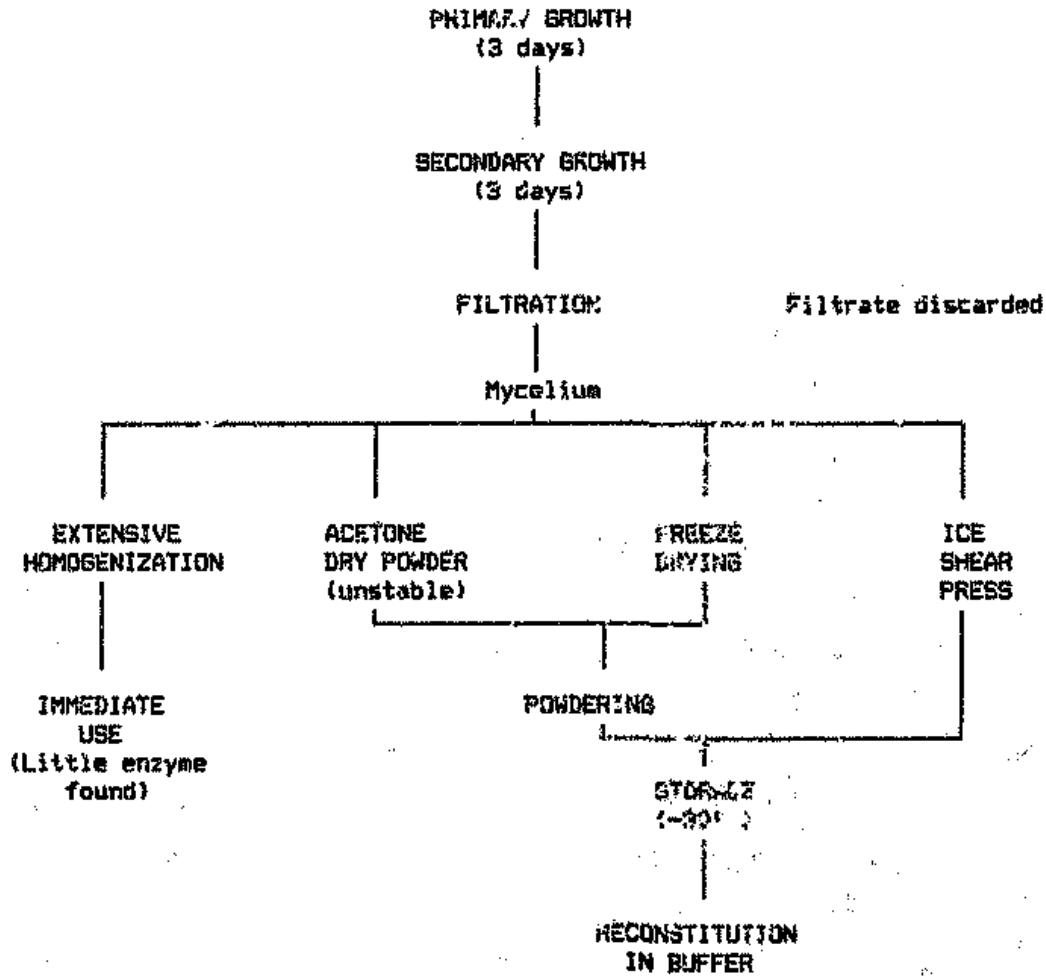
One of the reasons ascribed to the instability of these enzymes was the possible dissociation of the secondary, tertiary and quaternary structure by the high pressures used in the ice shear press. Herman (1985) reported that the preparation of an acetone-dry ice powder successfully circumvented this problem. Later, when the instability of the enzymes was found to be only indirectly linked to the method of mycelial rupture, a comparison of the ice shear press method of McGrath (1972) and the acetone dried powder method, together with the freeze drying method, was done.

All procedures were carried out at 4°C unless otherwise specified. Initially three methods of mycelial rupture were attempted before freeze drying was selected as the preferred method of mycelial rupture for the purification of the enzymes. Before a freeze dryer became available the preparation of acetone dried powders and the homogenization of the mycelium were used. Later it became feasible to freeze dry the mycelium and this method of mycelial rupture was investigated. In all three comparisons of the mycelial rupture methods were done - firstly the veracity of an acetone-dried powder was compared to homogenisation; secondly the acetone drying and freeze drying methods were compared and thirdly these last two methods were compared to the ice shear press method of rupture under conditions almost identical to those used by McGrath et al (1976, 1977). Figure 5 outlines the methods used.

2.3.2.1 HOMOGENIZATION

The secondary innoculum, which had been grown for two days, was removed from the shaker and the mycelium separated from the culture medium by filtration in a buchner funnel. Two culture flasks were used for the homogenization. The mycelium was washed with 500 ml 10 mM Tris HCl, pH 7.5 and 3 mM DTE and then homogenized for two minutes using an Ultra Turrex homogenizer. The homogenate was centrifuged (110 000g for 1 hour) and the supernatant, which constituted the crude preparation, assayed for protein and enzyme activity. The filtrate was also assayed for the presence of the

FIGURE 5 : A schematic outline of the methods of mycelial rupture used for the preparation of FPP synthase and δ-CPA synthase.



enzymes. Two samples, one which was homogenized and not centrifuged and the other which was centrifuged and not homogenized, were included for comparison. At no stage in this method of mycelial rupture was the mycelium stored pending further processing.

2.3.2.2 ACETONE DRY POWDER

The secondary innocula, grown as in 2.3.1 above, were stopped on the second day of growth. The mycelia and culture media were separated by filtration and the mycelia placed in acetone precooled to -70°C with dry ice. The mycelia were allowed to warm to 4°C (about 2 hours) and the acetone filtered off. Initially a double wash of the mycelium was done in order to ensure the removal of any residual water. However a comparison of the single and double wash preparations was done and it was found that the enzyme unit yield of a double wash was as little as half that of a single wash. As a result the single wash was selected as the method of choice. The mycelia were dried under vacuum for 2 hours and then ground to a coarse powder with a pestle and mortar. The powder was stored at -30°C where it was stable for more than 12 months.

The acetone dried mycelial powder (250 mg) was reconstituted by the addition of reconstitution buffer (10 mM Tris HCl, pH 7.5; 1 mM DTE) followed by brief stirring with a spatula. The reconstituted mycelium was allowed to stand for two hours after which it was centrifuged at 110 000g for 1 hour. The pellet was discarded and the straw-coloured supernatant filtered through Whatman No 4 filter paper. The filtrate constituted the crude preparation and was used for further processing and for enzyme activity and protein assays.

2.3.2.3 FREEZE DRYING

The cultures, grown as in 2.3.1 above, were removed from the shaker on the second day of the secondary innoculum. The mycelium and the culture medium were separated by filtration and the mycelium washed in the buchner

funnel with the reconstitution buffer (10 mM Tris HCl, pH 7.5, 1 mM DTE). The mycelium was then freeze dried over a two to three day period in a Virtis SRC 10 freeze dryer. Care was taken to prevent the mycelia thawing and thereby risking damage to the enzymes during the process of freeze drying. The dried mycelium was ground to a fine powder using a Phillips coffee grinder and stored at -30°C.

The powdered mycelium (250 mg) was reconstituted in reconstitution buffer in the same manner as the acetone dried powder (2.3.2.2) and centrifuged at 110 000g for 1 hour. The pellet was discarded and the supernatant filtered through Whatman No 4 filter paper to remove any lipid material. The filtrate was used as the crude preparation.

2.3.2.4 ICE SHEAR RUPTURE

The cultures, grown as in 2.3.1 above, were removed from the shaker on the second day of the secondary innoculum. The mycelium and the culture medium were separated by filtration and the mycelium washed in the buchner funnel with 10 mM Tris HCl, pH 7.9, 2 mM β-mercaptoethanol and 1 mM EDTA. The mycelium was then shaped in the ice shear press and frozen in dry ice (McGrath, 1972). A pressure of 200-300 psi was applied to the press and the press allowed to thaw. When the mycelium had reached a critical temperature the pressure forced the semifrozen mycelium through the sieve at the end of the press and the mycelium was thus ruptured. The ruptured mycelium was then refrozen in dry ice.

Two grams of sheared mycelium were thawed and stirred in four volumes of 10 mM Tris HCl, pH 7.9, 2 mM β-mercaptoethanol and 1 mM EDTA for an hour. The mycelium was then centrifuged at 110 000g for 1 hour and the pellet discarded. The supernatant was filtered through Whatman No 4 filter paper and the filtrate used as the crude preparation.

2.3.3 Development of the culture medium

The nitrogen source of the standard culture medium was varied firstly to maximize the mass of the mycelium per culture flask and secondly to maximize the activity of the FPP and 4-CPA synthases. The nitrogen source of the standard culture medium was substituted in four experiments with various concentrations of corn steep liquor, mycological peptone, malt extract broth or sodium aspartate. Enhanced concentrations of sodium nitrate were also tested in these experiments. The nitrogen sources and their concentrations for these experiments are summarized in table 2.

A primary innoculum using the standard medium was grown for 3 days for each of the four experiments. The nitrogen sources of the secondary innocula were changed to those in table 2. For all four experiments the growth of the secondary innocula was for five days. The filtrates of the cultures for the third experiment were extracted with acidified chloroform and read against chloroform at 280 nm to quantify the amount of α- and β-cyclopiazonic acid produced by the organism (log ε=4.31 at 280 nm). Aliquots from the chloroform extracts of the filtrates of the sodium nitrate-based culture medium (12, table 2), sodium aspartate-based culture medium (17) and both mycological peptone culture media (14 & 15) were run on a Merck F_{254} silica gel HPTLC plate. The HPTLC plate was developed in a system of ethyl acetate : methanol : ammonia (conc) 20:5:3 (v/v/v). The plates were sprayed with van Urk's reagent (1 g 4-dimethylamino benzaldehyde in a solution of HCl (conc) : 96% ethanol (1:3)) to detect the presence of indoles.

2.3.4 Maximization of Enzyme Production

Two culture media with enhanced nitrogen sources, together with the standard culture medium, were used to grow cultures for this experiment. Both nitrogen enhanced culture media used 80 g/l glucose. One of the nitrogen enhanced culture media used 9 g/l sodium nitrate while the other used 10 g/l sodium aspartate as the nitrogen source. Apart from these

TABLE 2 : The growth of Penicillium cyclopium on culture media with varied nitrogen sources and in some cases enhanced carbon sources are outlined in the table.

	Nitrogen source	g/l	Media pH	Other detail
EXPERIMENT ONE				
1	Sodium nitrate	4.5	5.8	
2	Corn steep liquor	3.0	6.0	
3	Mycological peptone	3.0	5.9	
4	*Malt extract broth*	*20.0*	*6.2*	*glucose omitted*
EXPERIMENT TWO				
5	Sodium nitrate	4.5	5.6	
6	Corn steep liquor	5.0	5.1	
7	Mycological peptone	5.0	4.9	
8	Malt extract broth	20.0	5.0	glucose omitted
9	Malt extract broth(+)	20.0	5.0	60 g/l glucose added
10	Sodium aspartate	6.7	5.0	
EXPERIMENT THREE				
11	Sodium nitrate	4.5	5.0	
12	Sodium nitrate	6.0	5.0	
13	Sodium nitrate	9.0	4.9	
14	Mycological peptone	8.0	5.2	
15	Mycological peptone	12.0	5.3	
16	Sodium aspartate	9.0	5.5	
17	Sodium aspartate	12.0	5.6	
EXPERIMENT FOUR				
18	Sodium nitrate	4.5	5.6	
19	Sodium nitrate	6.0	5.6	80 g/l glucose added
20	Sodium nitrate	9.0	5.5	80 g/l glucose added
21	Sodium aspartate	10.0	5.6	80 g/l glucose added

Apart from the nitrogen source all media constituents were those of the standard culture medium unless otherwise specified. Where changes were made it the glucose concentration that was altered. Malt extract broth and mycological peptone were from Oxoid. Malt extract broth contains 15% mycological peptone by weight. Media pH was determined after sterilisation. All cultures were done in duplicate.

alterations of the nitrogen and glucose sources the constituents of the culture media were those of the standard culture medium (table 1).

A primary innoculum was grown for three days using the standard culture medium. Secondary innocula were made into culture flasks containing the three different nitrogen sources. Over and above the three different culture media examined, the effect of the length of growth of the secondary innocula was also examined. The cultures of the secondary innocula were stopped on days two through five. In other words a three by four matrix (culture medium by growth time) of growths was performed. The growths were removed and the mycelia prepared using the acetone dry powder method (2.3.2.2). A double acetone wash was used. 250 mg of each of the mycelia were reconstituted in 10 mM Tris HCl, pH 7.5, 3 mM DTE, 10% (v/v) glycerol for 2 hours and then centrifuged at 110 000g for 1 hour. The supernatant was assayed using the coupled assay to determine the activity of the enzymes present in the reconstituted mycelium.

2.4 STABILITY OF THE FUNGAL ENZYMES

After determining that up to 60% of the enzyme activity of a crude preparation was being lost over a 24 hour period, an investigation was instituted to maximize the stability of the crude enzyme preparation. The investigation focused on the elements of the reconstitution buffer in order to establish firstly whether one of the elements was destabilizing the enzyme preparation and secondly to optimize the buffer's elements. The reconstituted mycelial preparations were stored at 4°C for the duration of these experiments. The mycelial preparations were assayed 24 hourly for up to six or seven days depending on the residual enzyme activity found.

In all cases the mycelium was reconstituted in reconstitution buffer for two hours and centrifuged (110 000g for 1 hour) and the supernatant filtered off. The filtrate constituted the crude preparation for the stability experiments.

2.4.1 Sulfhydryl reductants

Reconstitution buffers with dithioerythritol (DTE) concentrations of 1 mM, 2 mM, 3 mM and 5 mM, and 2-mercaptoethanol (10 mM and 20 mM) were made. The other components of the buffer were 10 mM Tris HCl, pH 8.0 and 1 mM EDTA. Both primary and secondary innocula were grown using the standard culture medium. The mycelial powder was prepared using a single acetone-dry ice extraction. Samples of 250 mg of acetone-dried mycelial powder per buffer were reconstituted in the reconstitution buffer. The reconstituted mycelial preparations were assayed for enzyme activity on days 0, 1 and 2.

It was found that when the activity of the porcine isomerase fell over a period of time it could be completely restored by treating the enzyme for 30 minutes with the addition of 3 mM DTE (final concentration). The fungal enzyme reconstitution containing 3 mM DTE showed the greatest stability of the DTE reductants and was treated with an additional 3 mM DTE for 30 minutes on day 6. Both the treated and untreated samples were assayed for enzyme activity.

2.4.2 Divalent cations

Primary and secondary innocula were grown and prepared as for the sulfhydryl reductant experiment (2.4.1). The sample buffers, based on 10 mM Tris HCl, pH 8.0, 2 mM DTE and 1 mM EDTA, were supplemented with concentrations of 1 mM, 2 mM and 5 mM magnesium sulphate, 1 mM and 2 mM manganese sulphate and one buffer that contained 1 mM each of magnesium sulphate and manganese sulphate. The reconstituted mycelia were assayed for activity on days 0, 1, 2, and 3. The samples supplemented with 1 mM and 2 mM magnesium sulphate as well as the control were also assayed on day 6.

2.4.3 Chelating agents

Primary and secondary innocula were grown and prepared as for the sulfhydryl reductant experiment (3.4.1). Amounts of EDTA to final concentrations of 0 mM, 1 mM and 2 mM EDTA were separately added to a base buffer of 10 mM Tris HCl, pH 8.0 and 2 mM DTE. The reconstituted preparations were assayed for enzyme activity on days 0, 1, 2, and 3.

2.4.4 Glycerol

The examination of glycerol as a supplementary element of the reconstitution buffer occurred in two stages. Initially buffers containing 10%, 15% and 20% glycerol were used. Subsequently buffers with glycerol concentrations of 0%, 2%, 4%, 6%, 8% and 10% were used. Following from the results of the examination of chelating agents (2.4.3) the use of EDTA was discontinued in this and the following experiments.

For the first experiment primary and secondary innocula were grown and prepared in the same manner as the sulfhydryl reductant experiment (2.4.1). The basic buffer used for this experiment was 10 mM Tris HCl, pH 8.0 and 2 mM DTE. Individual buffers were supplemented with 10%, 15% and 20% (v/v) glycerol. All these concentrations were the final ones. Two additional samples were prepared for this experiment. These were firstly a 20% glycerol - 1 mM $MgSO_4$ supplemented buffer and secondly a sample that was reconstituted overnight rather than for two hours using the 10% glycerol supplemented buffer. The samples were assayed on days 0, 1, 2, 3 and 6. It was found that no significant advantage was gained by using more than 10% glycerol in the reconstitution buffer.

Following on from the results of this experiment, coupled with the increased viscosity of the buffer (due to the glycerol and the low temperature - 4°C), the basic buffer, 10 mM Tris HCl, pH 7.9 and 2 mM DTE was supplemented individually with 0%, 2%, 4%, 6%, 8%, and 10% (v/v) glycerol concentrations. Samples of the acetone-dried mycelium from 2.4.1

were prepared in the same manner as the first supplemental glycerol experiment. The enzyme activity of the reconstitution was assayed on days 0, 1, 2, 3 and 6.

2.4.5 Buffer pH

The reconstitution buffer was made up at different pH's and the stability of the reconstituted enzyme preparation at these pH's examined. The counter ion used was maleate since the buffering range of Tris HCl does not extend below 7.0. The pH's examined were 8.0, 7.5, 7.0, 6.5 and 6.0. The Tris concentration was 10 millimolar and 2 mM DTE and 10% (v/v) glycerol were added as well. Both the primary and secondary innocula were grown and prepared with the same method as the sulfhydryl reductant experiment (2.4.1). The reconstituted mycelial preparations were assayed for enzyme activity on days 0, 1, 2, 3 and 7.

2.4.6 Buffer ionic strength

During preliminary ion exchange experiments it was observed that the fungal enzyme appeared to be more stable at ionic strengths higher than 10 mM Tris. The effect of ionic strength on the stability of the reconstituted mycelium was therefore examined. Tris concentrations of 10 mM, 20 mM, 50 mM, 100 mM and 200 mM were utilised for this experiment. 1 mM DTE was added and the pH was adjusted to 8.0 with HCl. No glycerol was added to the buffers used in this experiment as none had been used in the ion exchange experiments. The mycelial powder used for this experiment was prepared using the method in 2.4.1 above. Assays for enzyme activity were carried out on days 0, 1, 2, 3 and 7.

2.5 PURIFICATION OF THE FUNGAL ENZYMES

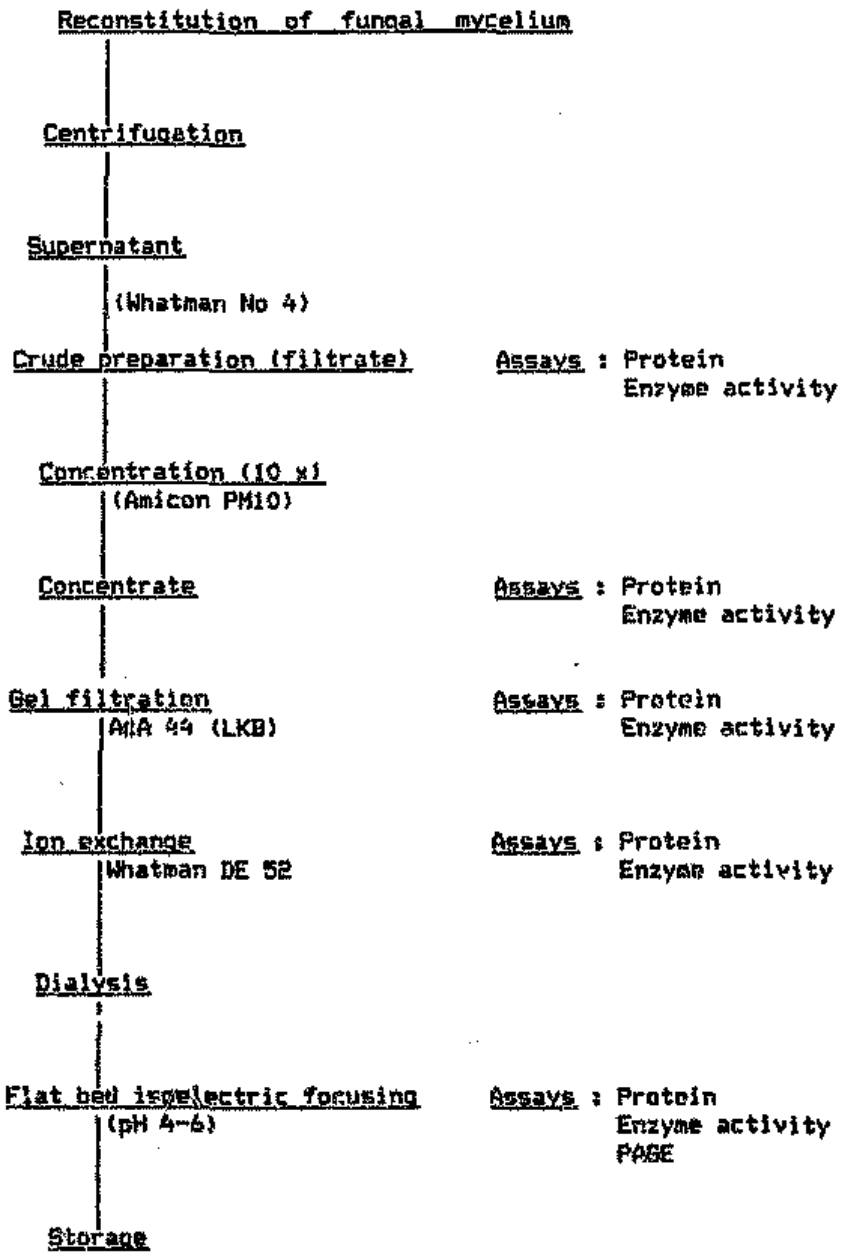
For the purification of the FPP and β-CPA synthases of Penicillium cyclopium a large stock of mycelium was prepared. The culture medium contained 10 g/l sodium aspartate as the nitrogen source as well as 80 g/l glucose. Otherwise the constituents of the culture medium were those of the standard culture medium (table 1). The growths were for 48 hours. The mycelia were freeze dried in a Virtis SRC 10 freeze dryer, powdered in a Phillips coffee grinder and stored at -30°C. Later when a preponderance of β-CPA synthase over FPP synthase was found in this stock, a second stock was prepared in an identical manner with the single exception that the growth was for only 36 hours. A comparison of the relative sizes of the peaks of these two enzymes from the 36 hours stock on elution from a LKB AcA 44 gel filtration column was found to have a more even distribution of the two synthases.

The freeze dried powder was reconstituted in buffer for purification. A schematic outline of the purification procedure is shown in figure 6.

2.5.1 Crude fractionation

Two and a half grams of freeze dried mycelium were reconstituted in 50 mM Tris HCl, pH 7.5, 2 mM DTE, 10% glycerol for 2 hours. The reconstituted mycelium was centrifuged at 110 000g for 1 hour at 4°C and the pellet discarded. The supernatant was filtered through Whatman No 4 filter paper to remove the lipids. The resultant filtrate was concentrated to a volume of less than 20 ml under nitrogen at 55 psi in an Amicon 52 concentrator using a PM10 membrane. Because an increased buffer viscosity is known to reduce the resolution on the gel filtration and ion exchange columns (Pharmacia, 1985) and since the buffer elements had been optimised to give an acceptable stability over a three day period (2.4), glycerol was excluded entirely from the buffer after the concentration step. Otherwise the buffer remained the same.

FIGURE 6 An outline of the purification protocol established for the FPP and β-CPA synthases from Penicillium cyclopium.



2.5.2 Gel filtration

The gel filtration media used was UltroGel AcA 44 which has a fractionation range of 10 000 - 130 000 daltons. The AcA 44 gel filtration column (80 cm x 2.6 cm) was equilibrated with 50 mM Tris HCl, pH 7.5, 2 mM DTE, for 24 hours before use. The sample (< 20 ml - 2.5.1) with a protein concentration in the region of 7-10 mg/ml was loaded. Blue dextran 2000 was used as a front marker and myoglobin (MW 17 600) as a back marker for the enzymes. The enzymes were fractionated on the AcA 44 column overnight at a flow rate of 18 ml/hour. Fractions of 13 ml were collected. The fractions were assayed for protein and for FPP and β-CPA synthase activity.

2.5.3 Ion exchange

The cation exchanger used was DEAE cellulose Whatman DE 52. 100 ml of ion exchange resin were prepared by stirring for 2 hours in 0.5 M HCl followed by 2 hours in 0.5 M NaOH. After the ion exchange resin's pH had been adjusted to 7.0, it was washed in 3 volumes of 100 mM Tris HCl, pH 7.0 followed by 6 volumes of 50 mM Tris HCl, pH 7.0. A 11 cm x 2.6 cm column was poured and equilibrated with 50 mM Tris HCl, pH 7.0, 2 mM DTE buffer, for two to three hours prior to use.

The active fractions from the gel filtration purification step (2.5.2) of all three enzymes were pooled and loaded onto the ion exchanger. The loaded column was washed. The wash was monitored with a LKB Uvicord 4701A and the wash continued until the absorbance had returned to its base level The enzymes were eluted using a linear gradient of 100 mM - 400 mM Tris HCl, pH 7.5, 2 mM DTE Fractions of 7 ml were collected. Protein remaining on the column was eluted using a 400 mM KCl solution. The fractions from the gradient were assayed for protein and for FPP and β-CPA synthase activity. The active fractions were pooled and dialysed against 2 mM Tris HCl, pH 7.5, 2 mM DTE. If the preparations were stored for longer than overnight an

equal volume of glycerol was added to the individual fractions (final concentration 50%) and the fractions stored at -30°C.

2.5.4 Isoelectric focusing

A flatbed apparatus was constructed to fit a 30 cm BioRad electrofocusing plate. The method used was essentially that recommended by BioRad for their apparatus (1982). Bio-Lyte electrofocusing gel was prepared by swelling 10 g of resin in distilled water for 2 hours and then washing the resin in 10 litres distilled water. The gel was manipulated as a 100% slurry and allowed to settle overnight. 100 ml of gel per run was used. Initially a broad pH range of LKB ampholytes was used (pH 3.5-10). Once the pI's had been established, ampholytes with a pH range of pH 4.0 - 6.0 were used. The ampholytes were used at a 2% concentration (5 ml per 100 ml gel of LKB 40% ampholytes). Six wicks were cut to size and were wetted with distilled water and inserted across the ends of the gel bed. These wicks enabled excess moisture to be wicked off from the gel once it had been poured. The ampholytes were mixed with the electrofocusing gel slurry which was then degassed. The slurry was poured into the level focusing plate. Excess moisture was wicked off at the electrode ends until the surface of the gel at the ends of the gel had a granular appearance.

The sample used for isoelectric focusing was either a crude preparation of the 36 hour stock or the relevant active fractions from the ion exchange step (2.5.3). Initially problems were experienced in implementing the technique with less than 1% of the protein loaded onto the gel being recovered after focusing. As a result crude preparations were used for focusing in order to establish the methodology prior to the subsequent focusing of the relevant fractions from the ion exchange eluent.

The sample was prepared prior to focusing by dialysis against 2 mM Tris HCl, pH 7.5, 2 mM DTE. If the volume was in excess of 2-3 ml the sample was concentrated at 25 psi in an Amicon 52 concentrator using a PM10 membrane prior to loading. After an aliquot had been taken for enzyme

activity and protein determination the sample was loaded in the centre of the plate using the BioRad sample applicator. Further wicking was applied to drain excess moisture from the gel until the whole bed had a granular appearance. Three or four of the wicks at each end were removed and replaced with a single wick soaked in the appropriate electrolyte. At the cathode the electrolyte was 1 N NaOH and at the anode it was 1 N H_3PO_4. The plate was inserted into the precooled (7°C) electrofocusing tank. The electrodes were positioned on top of the wicks that had been soaked in electrolyte. The bed was run at a constant 10 W (1000V maximum) for between 12 and 16 hours using a Shandon Vokam 2000-300-150 power source. As the ampholytes formed the pH gradient, the resistance of the gel increased. As a result the current fell and the voltage increased from an initial value of 450 V to a maximum which was set at 1000V. Initially focusing times of 16 hours were used but once it had been established that only 12 hours was necessary to focus the proteins this became the preferred time.

After the proteins had been focused, the power was switched off and two mylar strips inserted perpendicularly into the gel - one in the middle and one at the side. Once the mylar strips were wetted they were removed and dried immediately with a hairdryer to avoid lateral diffusion of the protein bands. No protein was visible when the mylar strips were examined under UV so the strips were sprayed with ninhydrin to detect the presence of any protein present. This sharpness of the bands on the mylar strips were a good indication of the success of the run.

Without wasting time the wicks were removed and a fractionating grid was inserted into the bed. The end of the grid was flush with the cathodic side of the gel bed. The gel fractions were scraped out using a micro spatula and 1.5 ml of distilled water mixed with each fraction. The pH was determined and 1 ml of 100 mM Tris HCl, pH 7.5, 2 mM DTE added to each fraction. This addition of buffer brought the pH to within 0.2 pH units of 7.5 in all cases. The fractions were assayed for enzyme activity and the protein concentrations determined. The active fractions were then eluted on a Sephadex G-25 column (30 cm x 9 cm) that had been previously

equilibrated with 50 mM Tris HCl, pH 7.5, 2mM DTE. The enzymes were collected in the void volume. Glycerol was added to the purified fractions to a final concentration of 20% (v/v).

2.5.5 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used as a qualitative means to establish the purity of the enzymes. A vertical slab gel apparatus was used together with a Vokam Shandon Southern V500/150 power pack. The two glass plates were cleaned with hexane and ethanol to be free of vaseline and grease. Two perspex spacers for the sides and one for the bottom were greased in vaseline and clamped between the glass plates to form a sealed unit. The gel solution, consisting of 12.5 ml 30% acrylamide, 2.2 ml 1% bisacrylamide, 6.2 ml 1.5 M Tris, pH 8.7, and 3.8 ml distilled water was mixed and degassed. 10 µl TEMED and 0.1 ml 10% ammonium persulphate were added to the gel solution and mixed. The gel solution was carefully poured into the prepared plates and a ½ ml layer of distilled water gently laid over the top. When the gel had polymerized (45 - 60 minutes) the water was drained off. 12 µl TEMED and 20 µl were mixed with 4 ml of the stacking gel stock solution (consisting of 10.0 g acrylamide, 270 mg bisacrylamide, 3.0 g Tris adjusted to pH 6.8, and made up to 200 ml with distilled water) and then degassed. The loading comb was inserted at the top between the two plates and the stacking gel was poured in between the comb and the gel.

After the stacking gel had polymerized the comb and bottom spacer were removed. The plates were then clamped into the tank. Running buffer (144 g glycine and 30 g Tris in 1000 ml) was diluted 1 : 9 with distilled water and then poured into the upper and lower buffer chambers. 10 µl or 20 µl of sample were mixed with an equal volume of glycerol. This was heated at 90° for 5 minutes. The sample was loaded using a micropipette. The anode was in the upper chamber and the cathode in the lower one.

The gel was run at either 150 volts for 5 hours or 40 volts overnight. Bromophenol blue was used to indicate how far the samples had run. When

the bromophenol blue indicator had reached the bottom the power was switched off. The plates were removed from the apparatus, separated and the gel dislodged. The gel was stained for 90-120 minutes in a solution of Coomassie Blue (1 g Sigma Brilliant Blue G in a solution of 1000 ml water : 500 ml methanol : 75 ml glacial acetic acid). The gel was destained in destaining solution (1000 ml methanol and 150 ml acetic acid diluted to 2000 ml with distilled water) until the protein bands were clearly visible. The destaining solution was changed 2-3 times.

2.5.6 Enzyme storage

As 50% glycerol does not freeze at -30°C the enzymes were stored as 50% glycerol solutions in 2 mM DTE at -30°C.

2.5.7 Molecular weight determination

The molecular weights of the FPP synthase and β-CPA synthase molecules were estimated using the Ultrogel AcA 44 gel filtration column. Molecular weight markers were purchased from Sigma. The markers used were alcohol dehydrogenase (150 000), bovine serum albumin (66 000), egg albumin (45 000) and carbonic anhydrase (29 000).

Masses of around 3 mg of the markers, together with blue dextran 2000 (Sigma), were dissolved in 2 ml of distilled water. A small amount of glycerol was added to increase the viscosity of the solution and thereby ensure a more uniform load on the column. The column was loaded and run overnight at a flow rate of 15 ml/hr. Fractions of 3½ ml were collected and read at 254 nm and 226 nm to determine protein. Samples of enzyme were run under identical conditions directly after the marker proteins and the active fractions determined by assaying for their activity.

The log10 of the molecular weight was plotted against the ratio of F/F_0 where F is the fraction number and F_0 is the number of the fraction where the blue dextran eluted from the column. A calibration curve was constructed and the molecular weights of FPP synthase and β-CPA synthase then read off the calibration curve.

CHAPTER THREE

PRELIMINARY RESULTS

3.1 GENERAL

3.1.1 Enzyme activity assays

Various problems were experienced in ensuring that the assay results reflected the true situation apropos enzyme activity. These problems were firstly in distinguishing between IPP-DMAPP isomerase and FPP synthase in the absence of a gas chromatograph with a detector capable of detecting either of the products of the enzymes at the concentrations used in the assays, and secondly in the assay of β-CPA synthase. Radioactively labelled ^{14}C DMAPP could not be purchased commercially and bulk preparation and storage of DMAPP was inadvisable since it was unstable even when frozen at -30°C. Therefore the porcine liver isomerase (2.2) was used to provide immediate supplies of DMAPP for the assays. Since the assays for the purification needed only to be relative, any discrepancies in the absolute amounts of DMAPP present were of little consequence so long as there was an excess of DMAPP present.

The method used to distinguish between FPP synthase and isomerase was as follows. Both enzyme preparations were assayed at pH 7.6 and at pH 6.0. The conditions of the assays were in all other respects the same. The enzyme activities of the assays were compared. It follows from the pH optima reported for these two enzymes (McGrath et al, 1977) that, where the change from pH 6.0 to pH 7.6 resulted in an increase in the enzyme activity, the enzyme was FPP synthase and where the converse was found, the enzyme was the isomerase. This method enabled the relative positions of the enzymes on elution from the ion exchange and gel filtration columns to be determined. Where pure FPP synthase was assayed the actual enzyme units measured was in fact half that observed since two labelled IPP moieties combine with one unlabelled DMAPP moiety.

The assay for the β-CPA synthase was modified from that of McGrath et al (1975, 1977). Instead of silica tlc plates being run or the samples being steam distilled, they were loaded onto Whatman 3MM dicks and the volatile alcohols evaporated in a stream of hot air from a hair dryer.

Comparative studies were done to determine whether this modification gave similar results to the silica plate method of McGrath et al (1976) and the results were always found to be within 4 % of each other.

McGrath et al (1977) reported that β-CPA synthase was not susceptible to denaturing by EDTA but rather was susceptible to manganese sulpnate while the converse was true for the isomerase. FPP synthase was susceptible to both EDTA and to a lesser extent manganese sulphate. A report by Cress et al (1981) reported another secondary metabolic enzyme, dimethylallyl pyrophosphate : L-tryptophan dimethylallyltransferase (from Claviceps sp. SD58), that shows a similar lack of susceptibility to EDTA.

An attempt was to establish whether this lack of inhibition of β-CPA synthase could be reproduced and whether it could then be used to advantage in the assay of β-CPA synthase. In the β-CPA synthase assay the addition of EDTA to the porcine IPP-DMAPP isomerase after it had been allowed to equilibrate would obviate the need to inactivate the porcine isomerase by boiling prior to the addition of the fungal β-CPA synthase being assayed. Furthermore EDTA would negate any effect that contaminating fungal isomerase might have on the amount of DMAPP available for the β-CPA synthase. The standard assay procedure for β-CPA synthase was used (2.1.1) with the exception that inactivation by boiling of the porcine isomerase was initially replaced by the addition of various concentrations of EDTA. Later boiling the porcine isomerase was used in addition to the addition of EDTA. Thus initially EDTA was intended to replace boiling in order to inactivate the isomerase. The rationale behind the use of EDTA was so that the pool of DMAPP produced by the porcine isomerase would not increase through the action of contaminating fungal isomerase during the period of the β-CPA synthase assay and thereby lead to dissimilar DMAPP concentrations in the respective assay tubes.

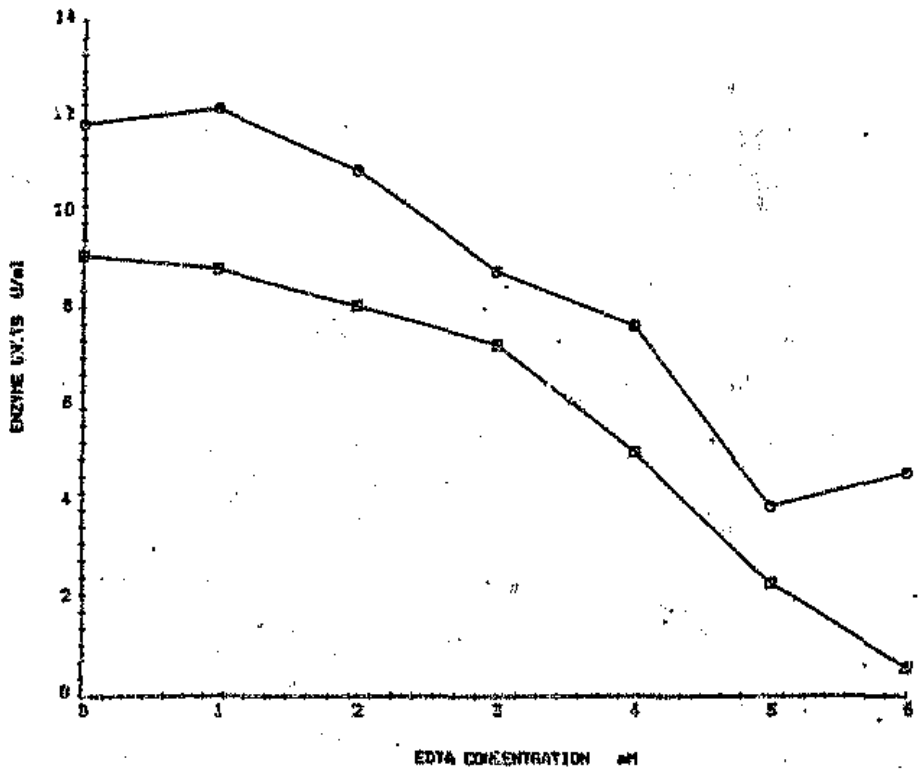
Because 5 mM magnesium sulphate was present in the assay mixture concentrations of 0 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM and 6 mM EDTA were examined. In other words only the 6 mM EDTA sample effectively had an excess of EDTA (≈ 1 mM) while, apart from the 5 mM EDTA sample, the other samples all had sufficient $MgSO_4$ to activate the isomerase. The results, shown in table 3 and figure 7, show a gradual decline on

TABLE 3 : The effect of varied EDTA concentrations on the assay for β-CPA synthase activity.

EDTA concentration	Enzyme units /ml
NO HEAT TREATMENT	
0 mM	11.85
1 mM	12.16
2 mM	10.88
3 mM	8.77
4 mM	7.64
5 mM	3.92
6 mM	4.59
HEAT TREATMENT	
0 mM	9.[illegible]
1 mM	8.84
2 mM	8.07
3 mM	7.26
4 mM	5.04
5 mM	2.33
6 mM	0.57

Once the isomerase had reached equilibrium the reaction mixture was heat treated by immersion in boiling water for a few minutes to inactivate the isomerase. Then the mixture was aliquoted out and EDTA to the above concentrations added. cAATrp was then added and the mixture used to assay the β-CPA synthase.

FIGURE 7 : A plot of the effect of a varied concentration of EDTA in the assay buffer for β-CPA synthase activity. The two experiments shown are firstly where the isomerase was deactivated with heat prior to the addition of the β-CPA synthase (□) and secondly where the heat treatment was omitted and the EDTA relied on to inactivate the isomerase (○).



increasing EDTA concentration. Because of the presence of 5 mM $MgSO_4$, only the 5 mM and 6 mM EDTA samples are pertinent to this discussion. In fact with the 1:1 ratio between EDTA and $MgSO_4$ the results mimic the $MgSO_4$ titration curves reported by McGrath et al (1977) for the fungal form, and Holloway and Popjak (1968) and Banthorpe et al (1977) for the porcine form of the isomerase. Examination of the 5 mM EDTA (effectively no free EDTA or $MgSO_4$) and 6 mM EDTA (effectively 1 mM free EDTA) samples in the table (no heat treatment) show a slight increase (3.92 to 4.59) when free EDTA becomes available. This increase as free EDTA became available was found reproducibly when immersion in boiling water was not used but it was never observed when heat inactivation was used. In order to exclude the possibility of more DMAPP being produced by isomerase that may not have been inactivated, a second experiment was done. In the second experiment the porcine isomerase was boiled after the addition of the EDTA. The assay mixture was cooled to 30°C after which β-CPA synthase was assayed. With the exception of the increase from 5 mM EDTA to 6 mM EDTA the results were similar to those obtained without heat inactivation (table 3).

It is clear that the addition of free EDTA did show an increase in the amount of β-CPA produced and therefore the activity of the β-CPA synthase did increase. Why however this enhancing effect of free EDTA was lost when the porcine isomerase was inactivated by boiling is unclear. Care was taken to ensure that the boiled reaction mixture had returned to 30°C before the addition of the β-CPA synthase so any detrimental effect on the β-CPA synthase was prevented. Furthermore no ready explanation can be offered for the higher enzyme units for EDTA concentrations 0 mM - 4 mM than that of 6 mM EDTA which is meant to favour the β-CPA synthase. Repetition of the assays in the complete absence of any cations together with a well defined DMAPP (i.e. a pure synthetic form) would be desirable to verify whether or not β-CPA synthase is susceptible to EDTA. However in the context of this investigation the inhibition of the isomerase by EDTA was not proven and therefore not incorporated in the β-CPA synthase assay procedure.

3.2 PURIFICATION OF PIG LIVER ISOMERASE

The purpose for initially purifying the porcine liver enzymes was two fold. Firstly because a source of IPP-DMAPP isomerase was needed to convert IPP to DMAPP for the β-CPA synthase assay. Only partial purification of the isomerase was necessary for the purpose of these assays since only factors that could reduce the concentration of DMAPP would interfere with the assay procedure. Thus the isomerase preparation had only to be free of FPP synthase and phosphatase activity. Holloway and Popjak (1967, 1968) assayed for phosphatase and at no stage found it to be a consideration apropos their isomerase assays. The contaminating FPP synthase on the other hand would have reduced the DMAPP concentration by combining it with an IPP unit to form GPP and then with another IPP unit to form FPP, the final product of FPP synthase. Therefore the isomerase had to be free of FPP synthase. Porcine liver was used as the source of isomerase rather than a fungal source firstly because β-CPA synthase, which also utilises DMAPP, is absent in porcine liver and secondly because the porcine liver isomerase is both more stable and has a higher enzyme yield per gram of starting material than the isomerase of fungal origin.

The second purpose for using porcine liver as a source of enzyme was to establish a purification protocol for the subsequent purification of the fungal enzymes.

While the resolution on the AcA 44 gel filtration column was shown to separate the isomerase from FPP synthase, the ion exchange step was used as a further purification step for the isomerase as trace amounts of FPP synthase contaminated the isomerase preparation. A typical purification profile of pig isomerase is shown in table 4. The isomerase eluted from the AcA 44 gel filtration column immediately after haemoglobin which was used as an indicator of the isomerase's position. FPP synthase was found to elute just before haemolglobin. This is

TABLE 4 : A typical purification profile of porcine liver IPP-DMAPP isomerase.

Fraction		
Filtrate (coupled assay)		
Protein conc.	mg/ml	10.9
Enzyme units	U/ml	1.87
Specific act.	U/mg	0.17
Gel filtration		
Protein conc.	mg/ml	0.75
Enzyme units	U/ml	0.44
Specific act.	U/mg	0.59
Ion exchange		
Protein conc	mg/ml	0.12
Enzyme units	U/ml	0.14
Specific act.	U/mg	1.17
Storage concentrate		
Protein conc	mg/ml	0.14
Enzyme units	U/ml	0.15
Specific act.	U/mg	1.07

The gel filtration and ion exchange results reflect the peak fractions of the elution profiles. The storage concentrate is the stock isomerase that was used in the assay for the ß-CPA synthase.

consistent with the findings of Holloway and Popjak (1968). The separation of the isomerase and FPP synthase can be clearly seen in the elution profile of the AcA 44 fractionation (figure 8); FPP synthase is the first peak (tubes 12 - 14) and isomerase the second one (tubes 18 - 21).

Tubes 18 through 21 were pooled and loaded onto a DE 52 ion exchange column (11.5 cm x 2.6 cm). The enzymes were eluted with a linear gradient of 100 mM-400 mM Tris HCl, pH 7.5, 2 mM DTE (figure 9). The fractions were assayed for enzyme and protein activity. The active fractions of FPP synthase-free isomerase were pooled and the buffer exchanged on a Sephadex G-25 column (30 cm x 0.9 cm) equilibrated with 10 mM Tris HCl, pH 7.5, 2 mM DTE. The pooled fractions were concentrated two fold on a Amicon PM10 membrane, aliquoted into 500 µl fractions and stored in liquid nitrogen.

This concentrate formed the basis of the stock of isomerase that was used for the preparation of DMAPP from IPP in the assay of the fungal enzyme, β-CPA synthase. On storage the isomerase had a specific activity of 1.07 enzyme units/mg and a protein concentration of 0.14 mg/ml. The purification factor for the storage concentrate of the isomerase was 6.3. While reported values of homogeneous isomerase are considerably higher than this (Banthorpe et al, 1977), the isomerase was essentially free from FPP synthase activity. Notwithstanding the fact that the isomerase was only partially purified, after the separation from the FPP synthase it was stored in liquid nitrogen for long periods of time. Some loss of activity on storage did occur but this was found to be reversible by the addition of 2 mM DTE.

FIGURE 8 : The elution profile of porcine liver [illegible] on a AcA 44 gel filtration column (80 cm x 2.5 cm). Fractions of 13 ml were collected.

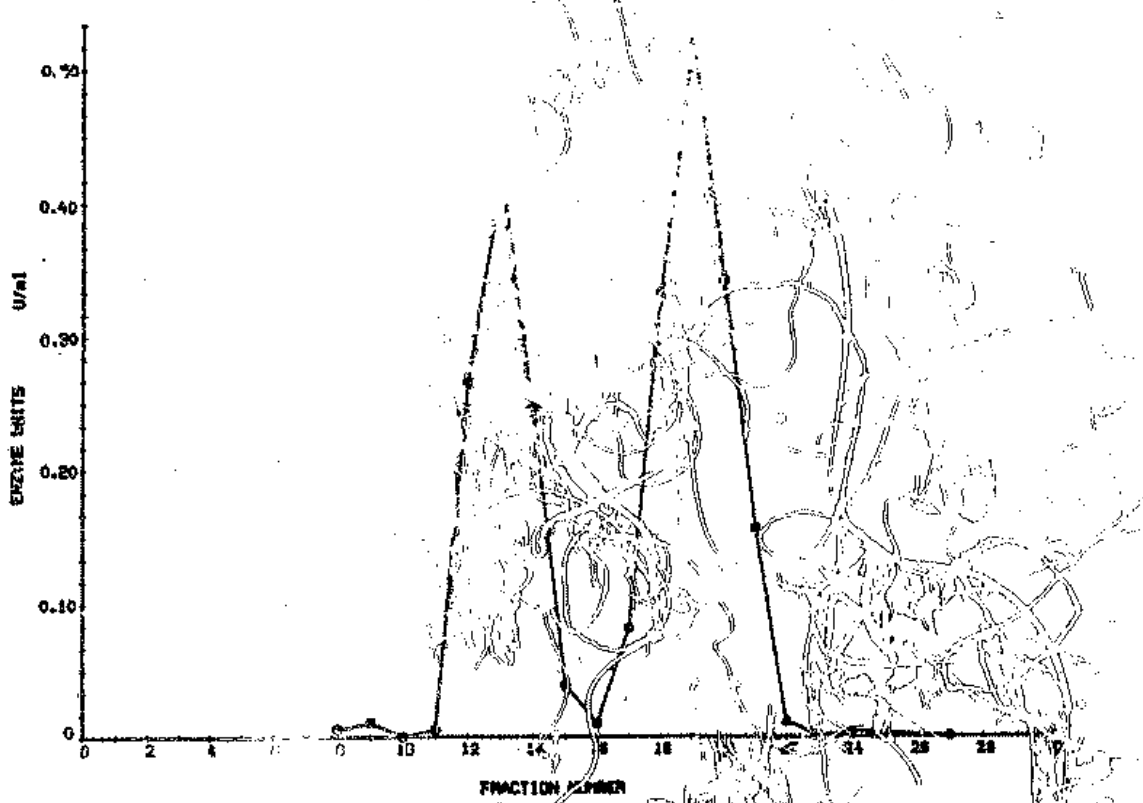
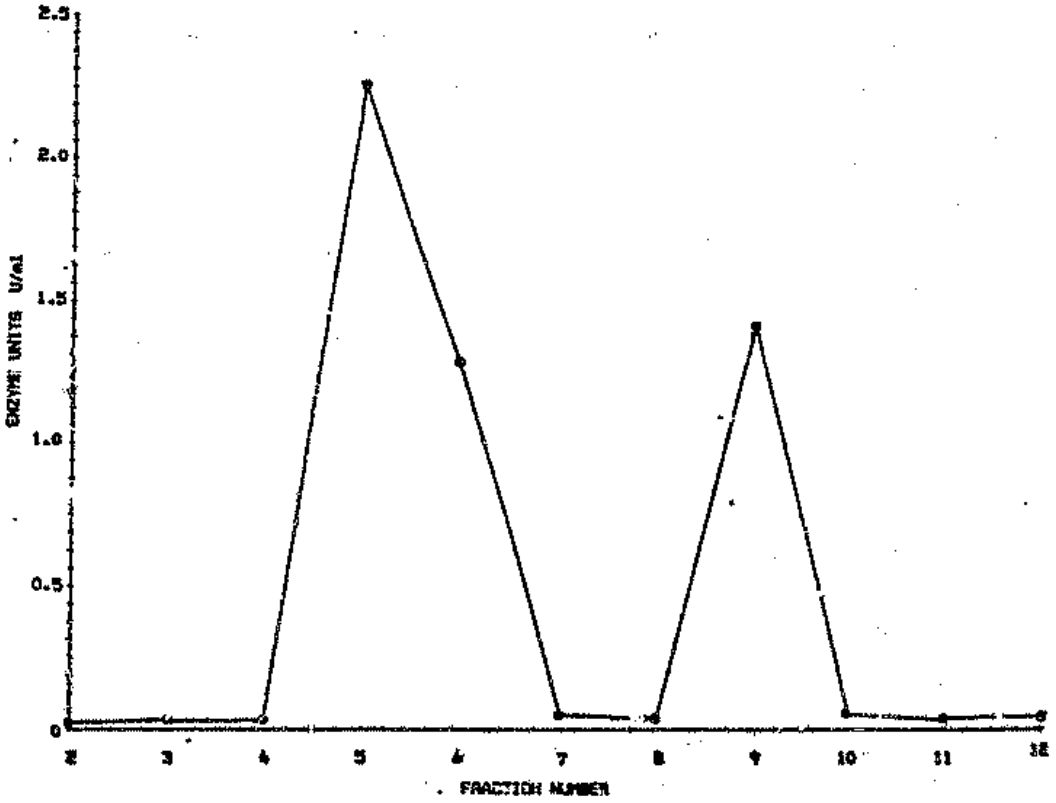


FIGURE 9 : The elution profile of porcine liver isomerase on a DE 52 ion exchange resin (20 m x 4 cm) using a 100 mM - 400 mM Tris HCl, pH 7.0, 1 mM DTE buffer. Fractions of 7 ml were collected.



3.3. THE ORGANISM : Penicillium cyclopium

3.3.1 Standard growth

Growth of the organism was achieved in a reproducible manner with less than 10 % variation between successive growths. Fungi, like other microorganisms, have a genome that mutates easily and it is possible for the pathway responsible for the production of a secondary metabolite to be switched off or lost entirely under certain circumstances. The starter cultures of Penicillium cyclopium that were to be used for the primary innocula were freeze dried agar slants stored in liquid nitrogen. To establish whether the freeze dried slants still had the facility to produce α- and β-cyclopiazonic acids, the slants were used to innoculate a growth using the standard culture medium. After five days the growth of the secondary innoculum was stopped and the mycelia and filtrates extracted with acidified chloroform. The extracts were run on tlc plates in an ethyl acetate : methanol : conc ammonia (20:5:3) system and were sprayed with van Urk's reagent. The tlc plates showed that both cyclopiazonic acids were produced and hence the freeze dried slants were used for starting the primary innocula.

3.3.2 Rupture of the mycelium

The filtrate from the secondary innoculum was assayed for enzyme activity using the coupled assay supplemented with unlabelled DMAPP. No enzyme activity was found in the filtrate. This finding is consistent with the findings of other workers (McGrath et al, 1977). It follows that the enzymes in question must be present in the mycelium. The presence of the cell wall surrounding the mycelium presented a problem. The cell wall, being far more rigid than the cell membrane, meant that a far harsher means of cell rupture had to be found for the fungal mycelium than was used for the porcine liver. McGrath et al (1976, 1977) used an ice shear press to rupture the mycelial cell walls. They however found that their

enzyme preparations were unstable. Furthermore they found that the protein themselves showed varying characteristics when analyzed using conventional protein chemistry methods. Herman (1985) on the other hand reported that the use of acetone-dry ice yielded a stable enzyme preparation.

Comparison of acetone dried mycelia with mycelia prepared by extensive homogenisation showed that five times more protein was derived from the acetone-dry ice method (part one, table 5). The enzyme units from the acetone dried mycelia as measured by the coupled assay were up to nine fold greater than those of the homogenate. Two points are interesting to note here. The first is that comparison of the protein concentrations suggests that homogenisation failed to successfully rupture the mycelium. The second point is that, while the overnight reconstitution produced 50 % more enzyme units than the 2 hour reconstitution, their protein concentrations are similar. This strongly suggests that the enzymes are associated with subcellular structures such as the cell membrane.

As a result the preparation of a dry ice-acetone powder was utilized to rupture the mycelial cell wall. Initially immersion in the acetone-dry ice mixture was repeated a second time to make sure all the water molecules had been removed from the mycelial preparation. However a comparison of single and double wash preparations showed one and a half times the yield of enzyme units for a single wash compared to two washes (part one, table 5). Hence for the acetone dried powder method a single wash was used exclusively thereafter.

Later however a freeze dryer became available and, as the reconstituted preparations from acetone dried powders were not sufficiently stable for purification of the enzymes, this method was also investigated as a means of rupturing the mycelial cell wall. Part two of table 5 shows a comparison of the acetone-dry ice and freeze drying methods of mycelial rupture. From part two of the table it can be seen that the freeze dried mycelia yielded more protein and more enzyme units than the acetone dried mycelia. The specific activity of the acetone dried powder method was significantly lower than the freeze dried (3.24 U/mg as opposed to 5.03

TABLE 5 : A comparison of methods of rupturing the fungal mycelium.

	Protein conc. mg/ml	Enzyme mUnits /ml	Specific activity mU/mg
PART ONE			
Acetone dried			
1 wash (1.01g)	0.51	2.06	4.04
2 washes (0.92g)	0.54	1.35	2.49
Homogentate centrifuged	0.11	0.23	2.09
Filtrate	0.20	0.0	0.00
Homogenate not centrifuged	0.21	0.22	1.05
PART TWO			
Acetone dried			
nitrate medium	0.65	2.20	3.38
aspartate medium	0.39	1.25	3.24
Freeze dried			
nitrate medium	0.84	3.20	3.82
aspartate medium	1.45	7.30	5.03

The masses given for the acetone dried mycelia are the dry weight yields of 8 growths (200ml) of culture medium.

U/mg) and so freeze drying, as a method of cell wall rupture, was the method of choice for the purification.

It became apparent during the investigation into the most optimal buffer constituents that the method of mycelial rupture had only an indirect effect on the stability of the fungal enzymes. Several other workers working with enzymes from fungal sources reported that the enzymes were markedly unstable in comparison to enzymes from vertebrate forms (Cress et al, 1981, Eberhardt and Rilling, 1975 and Lee et al, 1976). As a result a comparative study was undertaken in order to establish whether the ice shear press method used by McGrath et al (1976, 1977) was directly responsible for the unstable and variable characteristics found by them. The study was undertaken in two parts - firstly the stability of the crude preparation and secondly the eluent from a cation exchange resin, Whatman DE 52, were examined. In keeping with the conditions used by McGrath et al (1976, 1977) the sulfhydryl reductant used in this comparison was 2 mM β-mercaptoethanol. Together with β-mercaptoethanol 1 mM EDTA was used in the 10 mM Tris HCl, pH 7.9 buffer. The results are shown in table 6.

Unfortunately the pressures used by them could not be reproduced on the press and the mycelium was ruptured at between a third and a half of the pressure used by them (200-300 psi in place of 600 psi). The table shows the assay results of the crude preparation and the eluent from the DE 52 ion exchange. McGrath in a personal communication indicated that loss of activity on elution from the column was rapid and immediate. Hence both sets of assays were only done 6 hours after the enzymes had been eluted from the ion exchange. If a rapid loss of activity had occurred on elution from the ion exchange column then no activity would have been apparent after this time period. As activity was observed after the lag period this investigation failed to implicate directly the ice shear press method in the instability found by McGrath et al. Equally it failed to conclusively prove the converse. A major contributing factor in this investigation that would ne have to be eliminated is the lower pressures used. While 600 psi may damage proteins, there is nothing to suggest that 200-300 psi must necessarily follow suit.

TABLE 4 : A comparison of mycelial rupture using a ice shear press with those of preparing acetone and freeze dried powders. Two sources were used - the crude preparation and the eluent from a DE 52 ion exchange column. ND = not determined.

	Protein conc. mg/ml	Enzyme Units /ml	Specific activity mU/mg
CRUDE PREPARATION			
Freeze dried			
nitrate medium	0.84	3.20	3.82
aspartate medium	1.45	7.30	5.03
Acetone dried			
nitrate medium	0.65	2.20	3.28
aspartate medium	0.39	1.25	3.24
Ice shear press			
nitrate medium	0.65	2.20	3.38
aspartate medium	0.39	1.25	3.24
ION EXCHANGE ELUENT			
Freeze dried			
nitrate medium	ND	0.66	ND
aspartate medium	ND	0.68	ND
Acetone dried			
nitrate medium	ND	0.57	ND
aspartate medium	ND	1.02	ND
Ice shear press			
nitrate medium	ND	0.83	ND
aspartate medium	ND	0.78	ND

3.3.3 Development of the culture medium

Fungi have a very primitive form of homeostatic regulation to maintain a constant overall metabolic balance and consequently they tend to respond to the circumstances of their environment in a very direct way. They tend to grow at the fastest possible rate when there is a plentiful supply of all the essential nutrients. When these one or more of nutrients are depleted the basal metabolic rate decreases and the organism's metabolism becomes unbalanced as various primary metabolic pathways are slowed or in some cases reversed to provide the organism with the missing essential nutrients. Neethling and McGrath (1977) demonstrated this by measuring the depletion of lipid material over the course of a growth.

The feature of an uncontrolled rate of growth is that an inordinate amount of insoluble material is synthesized by the fungus in the form of cell walls and membranes. The immediate consequence of this is that for a given mass of fungus far less soluble protein is obtained than for example porcine liver. Consequentially a larger amount of mycelium is needed to obtain a sufficient quantity of the enzymes in question for their purification since these enzymes are soluble or at most weakly bound to the cell's inner membrane (McGrath et al, 1977). For this reason it was therefore desirable to maximize the mycelial mass per flask in order to increase the overall yield of the enzymes. While the specific yield of enzyme per gram of mycelium would in all likelihood remain the same, the increased mass would mean an increase in the total enzyme present in the mycelium.

Neethling and McGrath (1977) showed that the depletion of nitrogen in the culture medium was an important factor for switching the metabolism from the growth phase (trophophase), where the none of the nutrients are limiting and growth is exponential, into the cyclopiazonic acid production phase (idiophase) where the nitrogen source is depleted and the growth rate has slowed completely. It has been shown that if the nitrogen source is kept constant, increasing the concentration of the carbon source (in these experiments limited to glucose) favours the production of other

secondary metabolites at the expense of α-cyclopiazonic acid (Nover and Luckner, 1969, Holzapfel, 1980). While the aim of this section was to increase the mycelial mass yields, it was essential to ensure that α-cyclopiazonic acid production was not lost. Bearing in mind what has been mentioned apropos the nitrogen source, it is clear that the most suitable nutrient to manipulate in order to increase the mycelial mass yield was nitrogen. Thus in these experiments to increase the mycelial mass yield the nitrogen source was the only one that was altered - otherwise the culture medium used was the standard medium (table 1). Where the concentration of the nitrogen source was substantially increased (>30 % over that of the standard medium) it was assumed that the glucose concentration could also be increased by a similar amount without upsetting the nitrogen / glucose balance to any significant extent (see cultures 19 to 21, table 2 above).

A series of four growths was undertaken in order to improve the yield of mycelial mass per culture flask (i.e. 100 ml). This was done by varying both the concentration and the nature of the culture medium nitrogen source. The results of these four growths are summarised in tables 7 to 10.

Neethling (1972) reported that utilizing corn steep liquor as the nitrogen source for growth gave a ten fold greater yield of mycelial mass than that of sodium nitrate, the nitrogen source used in the standard culture medium. Herman (1985) also reported an increased mass of mycelium using corn steep liquor as the nitrogen source. However the batch of corn steep liquor used in these growths failed even to match the masses achieved with the standard culture medium (tables 7 & 8).

Two other general culture media for fungi, Oxoid mycological peptone and Oxoid malt extract broth, were also examined. Malt extract broth contains sufficient carbon source in itself and thus with one exception the growths using this nitrogen source contained no additional glucose. Despite this the malt extract broth-based cultures yielded about half the mass when compared to the standard culture medium (cultures 4, 8 and 9). The addition of glucose to culture 9 in growth 2 showed that the lack of

TABLE 7 : Mycelial mass yields of cultures grown with varied culture medium nitrogen sources - 1.

	Nitrogen source	g/litre	Media pH	pH after growth	Mass g/flask	Colour
1	Sodium nitrate	4.5	5.80	5.[illegible]	1.43	white
2	Corn steep liquor	3.0	6.04	4.74	0.41	light brown
3	Mycological peptone	3.0	5.94	4.05	0.81	beige
4	Malt extract broth	20.0	6.20	6.28	0.78	milky brown

Standard culture media apart from the nitrogen sources were used. Glucose was omitted from the malt extract broth culture. The malt extract broth and the mycological peptone were from Oxoid. The malt extract broth contained 15% mycological peptone by weight. All cultures were grown for 5 days and were dor[illegible] in duplicate.

TABLE 8 : Mycelial mass yields of cultures grown with varied culture medium nitrogen sources - 2.

	Nitrogen source	g/litre	Media pH	pH after growth	Mass g/flask	Colour
5	Sodium nitrate	4.5	5.58	6.27	1.43	white
6	Corn steep liquor	5.0	5.11	4.58	0.38	light brown
7	Mycological peptone	5.0	4.92	4.00	1.53	light yellow
8	Malt extract broth(-)	20.0	4.98	6.14	0.72	dark brown
9	Malt extract broth	20.0	4.97	6.92	0.60	yellow/brown
10	Sodium aspartate	6.7	5.04	5.20	1.44	white

Standard culture medium was used with the exception of the nitrogen source. The Malt extract broth(-) culture had no glucose added to the medium. All cultures were grown for 5 days and were done in duplicate.

TABLE 9 : Mycelial mass yields of cultures grown with varied culture medium nitrogen sources - 3.

	Nitrogen source	g/litre	Media pH	pH after growth	Mass g/flask	Absorb 280 nm	Colour
11	Sodium nitrate	4.5	4.98	6.31	1.22	0.324	beige
12	Sodium nitrate	6.0	4.95	6.48	1.59	0.395	beige
13	Sodium nitrate	9.0	4.92	7.05	1.68	0.335	beige
14	Sodium aspartate	9.0	5.50	6.27	1.69	0.320	beige
15	Sodium aspartate	12.0	5.56	7.17	1.73	0.310	mustard
16	Mycological peptone	8.0	5.19	4.02	1.48	0.258	beige
17	Mycological peptone	12.0	5.31	4.51	1.85	0.375	beige

The standard culture medium was used with the exception of the nitrogen source. The absorbance at 280 nm is that of the chloroform extract of the acidified culture medium at the end of the growth period. This estimation gives an indication of the possible presence of α- and β-CPA.
All cultures were 5 day growths and were done in duplicate.

TABLE 10 : Mycelial mass yields of cultures grown with varied culture medium sources and an enhanced concentration of 80 g/l of glucose.

	Nitrogen Source	g/litre	Glucose g/litre	Media pH	pH after growth	Mass g/flask	Colour
18	Sodium nitrate	4.5	60.0	5.60	6.40	1.45	clear white
19	Sodium nitrate	6.0	80.0	5.58	6.12	1.42	clear white
20	Sodium nitrate	9.0	80.0	5.53	7.20	2.05	milky white
21	Sodium aspartate	10.0	80.0	5.61	5.34	2.02	milky white

Standard culture media with the exception of the nitrogen and carbon sources were used. Cultures 2 to 4 were supplemented with an additional 20 g/l of glucose (80 g/l total concentration).
Estimation of the α- and β-CPA concentrations was not done.
All cultures were 5 day growths and were done in duplicate.

growth was not due to insufficient glucose. The contrary was observed in that when the malt extract broth was supplemented with glucose, the mycelial mass yield was decreased. Malt extract broth was discarded as a potential nitrogen source in the third and four growths.

The initial growth with mycological peptone as the nitrogen source failed to match the mycelial mass yields obtained with the standard culture medium (culture 3). In the second growth mycological peptone was used in comparable gram amounts to sodium nitrate and the growths gave equivalent mycelial mass yields (culture 7). In the third growth (table 9) the mycelial mass yields for the mycological peptone-based culture medium were high. However the complete absence of α-cyclopiazonic acid in the filtrate led to this nutrient being discarded as an alternate source of nitrogen in the culture medium.

Neethling (1972) also reported high mass yields for aspartic acid, serine and glutamic acid amongst other nitrogen sources that she tested. Aspartic acid in the form of sodium aspartate was examined as a potential nitrogen source in growths two, three and four. The masses obtained from growths based on sodium aspartate were in all cases similar to those of the equivalent mass of sodium nitrate. In the third growth the addition of an extra 3 g/l to the culture medium resulted in a minimal increase of mass and hence 10 g/l was selected as the concentration for the fourth growth.

For the third growth (table 9) the filtrates were acidified and extracted with chloroform in order to determine whether using the increased nitrogen sources the organism was still producing α-cyclopiazonic acid over the period of growth. The absorbances at 280 nm of the extracts are shown in table 9. The absorbances from the sodium aspartate-based culture media were slightly lower than those of the nitrate-based culture media while the mycological peptone-based growths showed similar but erratic absorbances. Samples of the filtrate extracts (30 µl) from the 6 g/l sodium nitrate-based (12, table 9) and the 9 g/l sodium aspartate-based (14, table 9) cultures as well as both of the mycological peptone-based cultures (8g/l and 12g/l) (16 & 17, table 9) were loaded on a HPTLC plate

and run in an ethyl acetate : methanol : conc ammonia (25:3:3) system. The plates were stained with van Urk's reagent to detect for the presence of indoles. The result is shown in figure 10. It is evident that there was no α- and β-cyclopiazonic acid present in either of the extracts of the mycological peptone-based cultures. α-cyclopiazonic acid was however present in roughly equal amounts in both the nitrate and aspartate-based culture media. Mycological peptone was thus discarded as an alternate nitrogen source.

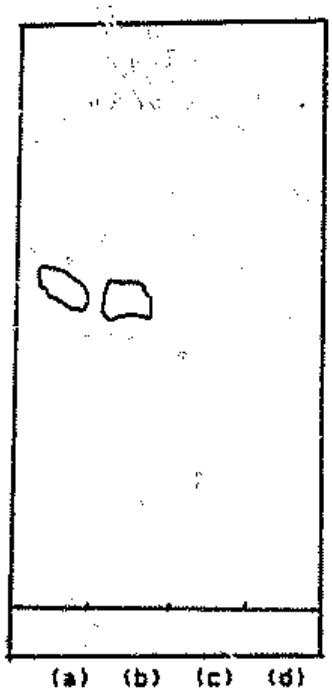
In the fourth growth (table 10) the two promising nitrogen sources, sodium aspartate and sodium nitrate were used in concentrations of 6 g/l and 9 g/l in the case of the former and 10 g/l in that of the latter. The glucose concentration was raised by 20 g/l to 80 g/l. The continued production of α-CPA in cultures grown with changed nutrient constituents having been established in the third experiment, no determination of cyclopiazonic acids was done in this experiment. The results are shown in table 10. It can be seen that the increased glucose led to roughly a 20 % increase in the mycelial mass per growth.

Having achieved a significant (40 %) increase in the mass yield, the enzyme yields of mycelia grown in sodium aspartate and enriched nitrate were examined firstly to ensure that the enzymes in question were still produced and secondly to determine the enzyme yields from these nitrogen sources.

3.3.4 Maximization of enzyme production

In section 3.3.3 it was shown that increased mycelial mass yields could be obtained by varying the nitrogen source present in the culture medium. It was also shown that this did not markedly affect the production of α-cyclopiazonic acid. However it was assumed that in the cultures grown with a different nitrogen source the specific yields of the enzymes per gram of mycelium would be similar to those of the standard medium-based cultures. The investigation in this section set out to establish

FIGURE 10 : A comparison of α-cyclopiazonic acid production on tlc plates from 5 day growths on culture media that had enhanced nitrogen sources. These nitrogen sources are (a) 6 g/l sodium nitrate-based (12, table 9), (b) 9 g/l sodium aspartate-based (14, table 9), and (c) 8 g/l and (d) 12 g/l mycological peptone-based cultures (16 & 17, table 9). The blue stain on the tlc plate is the positive van Urk's stain for the presence of indoles.



whether this was in fact a valid assumption.

Having found that enriched nitrate-based and aspartate-based culture media yielded increased mycelial masses per litre of culture medium, the yields of enzyme units were determined for these two culture media. The enzyme yield from the standard culture medium, which was used as the control, was also determined. The mycelia were reconstituted 10 mM Tris HCl, pH 7.5, 2 mM DTE and centrifuged and filtered to form the crude preparation. No further purification was carried out and the coupled assay was used to determine the relative enzyme activities.

The nature of the nitrogen source is in itself a factor affecting the rate of growth of the organism. Sodium nitrate is converted by the fungus into ammonia before it is utilized. Aspartate is converted to fumarate and ammonia through the action of aspartate ammonia-lyase. Given that over the period of the growth phase the ratio of enzyme to mycelial mass is likely to be fairly constant and that the rate of growth of the fungus could well vary with the nature of the nitrogen source, the yield of enzyme may vary with the length of time that the culture was grown for. To this end the day on which the concentration of enzyme was maximal was determined by carrying this study out over a four day period. The secondary innocula were removed on days two through to five for this purpose. The results are shown in table 11 and figures 11 and 12.

From table 11 it can be seen that the second day of the secondary innoculum produced the highest number of enzyme units. This is in agreement with McGrath et al (1977) who found the maximal enzyme concentrations after 56 hours of culture. For the aspartate-based growths preparations on day 3 had 24 % of the activity of day 2. The nitrate-based growths showed similar losses from day 2 to day 3. The fall in enzyme activity would be consistent with the switch from the growth phase to the idiophase at the time. In other words the nitrogen becomes rate limiting and the metabolic balance of the organism becomes unbalanced and protein is broken down. The rest of the discussion in this section will centre on the results of day 2.

TABLE 11 : The optimal production of 2, 3, 4 and 5 day growths of FPP and β-CPA synthases using enriched nitrogen c re media.

Culture Medium	Growth Period /days	Media pH	Mycelial yield g/100ml	Dpm /assay	Enzyme units U/ml
Sodium nitrate	2	ND	1.08	955	0.96
450mg per flask	3	ND	1.50	135	0.14
Glucose	4	5.93	1.45	100	0.10
6g per flask	5	6.38	1.44	117	0.12
Sodium nitrate	2	ND	0.96	1215	1.22
900mg per flask	3	ND	1.36	380	0.38
Glucose	4	7.02	1.77	320	0.32
8g per flask	5	6.87	1.94	120	0.12
Sodium aspartate	2	ND	0.95	2525	2.53
1000mg per flask	3	ND	1.28	617	0.62
Glucose	4	5.89	1.46	236	0.24
8g per flask	5	5.47	1.59	295	0.29

ND = not determined.
The mycelial yield is per 100 ml of culture medium. The assays were for 5 minutes and 200 µl of enzyme extract was used.
All samples were done in duplicate.

FIGURE 11: A plot of the enzyme activity of the enzymes from cultures grown over a four day period on culture media containing 4.5 g/l $NaNO_3$ (■), 9.0 g/l $NaNO_3$ (▲) and 10.0 g/l aspartate (●). The glucose concentrations of the last two cultures were 80 g/l each while that of the first culture was 60 g/l.

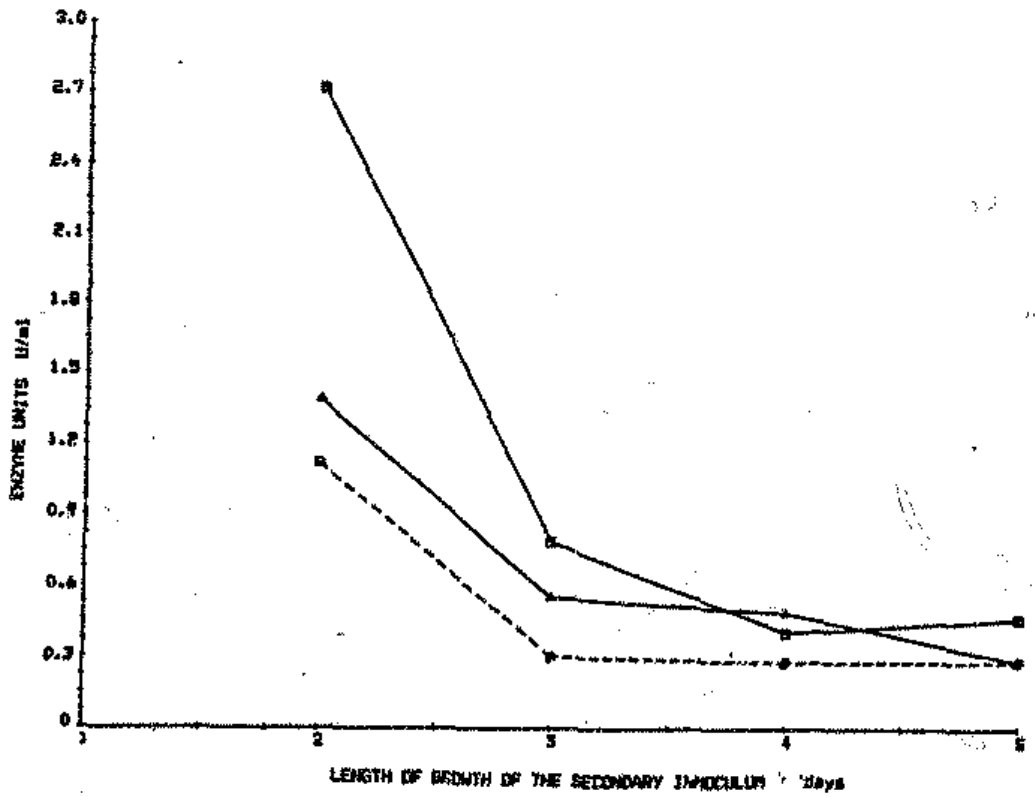
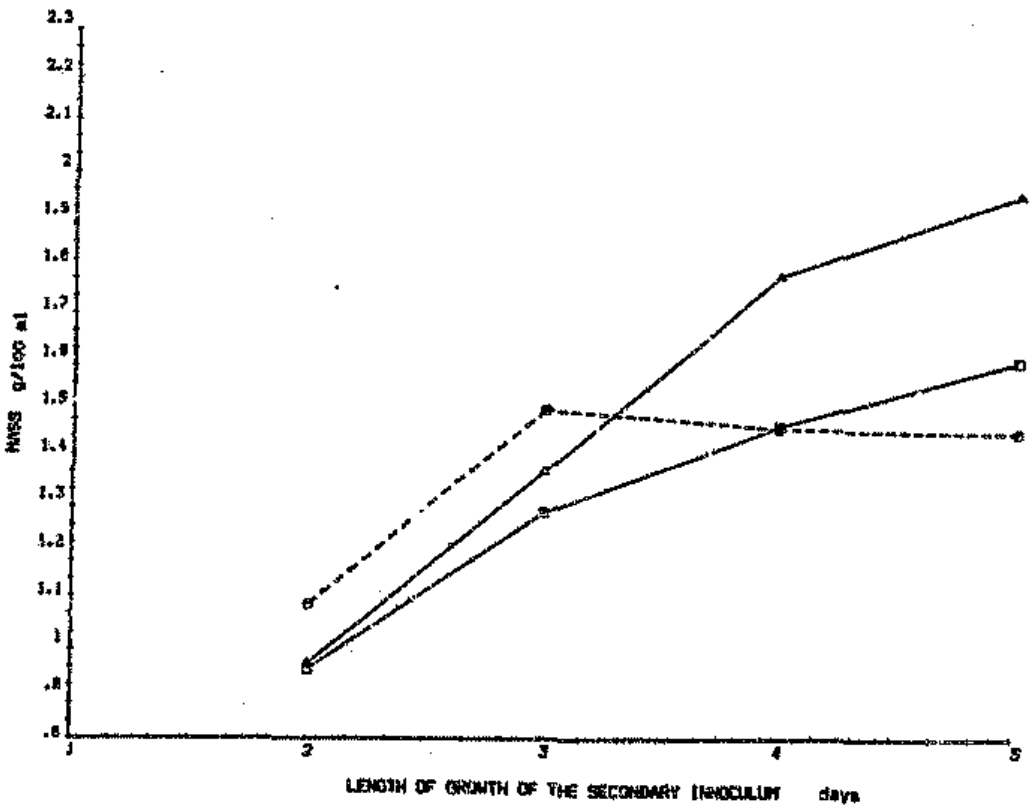


FIGURE 12 : A plot of the mycelial mass yields of cultures grown over a four day period on culture media containing 4.5 g/l $NaNO_3$ (o), 9.0 g/l $NaNO_3$ (▲) and 10.0 g/l aspartate (□). The glucose concentrations of the last two cultures were 80 g/l each while that of the first culture was 60 g/l.



It is also evident from the table and the figures that the aspartate-based cultures on the second day yielded twice the amount of enzyme compared to the enhanced nitrate-based cultures and two and a half times compared to the standard culture medium. The enhanced nitrate-based culture medium showed a parallel increase in enzyme yield over days 2, 3 and 4 when compared to the standard culture medium (figure 11). This is consistent with the assumption that while the enzyme would increase with increasing mycelial mass, the specific yield (U/g mycelium) would remain constant.

Once the aspartate-based culture medium had shown an increased yield of mass and specific enzyme yield, the stability of the aspartate-based culture medium was compared to that of the nitrate-based culture medium. It was found that reconstituted mycelium from the aspartate-based culture medium had a similar stability to the reconstituted mycelium from the nitrate-based culture medium. The comparable stability of the aspartate-based culture medium, together with its higher yields of enzyme, led to it being used for the bulk preparation of freeze dried mycelial powder for the purification of the enzymes. But before the enzymes were purified, their stability in reconstitution buffer had to be optimized.

3.4 STABILITY OF THE FUNGAL ENZYMES

The buffer initially used for the reconstitution of the acetone dried powder was 10 mM Tris HCl, pH 7.0, 1 mM DTE and 1 mM EDTA (Herman, 1985). The freeze dried powder used for enzyme purification did not yield uniform reconstitutions - nor were they sufficiently stable for the purification of the enzymes. In order to improve this for the purification process, the elements of the buffer were individually examined. The aim was to optimize the stability of the reconstituted mycelium and to determine whether any particular element of the buffer was destabilizing the reconstituted mycelial preparation. The minimum period in which the purification could be effected was three days and thus the

loss of activity over the three day period was taken as the criterion for the optimization of the reconstituted mycelium. All concentrations of the buffers used in these experiments were final concentrations. The mycelial preparation used in this section was grown on standard culture medium and prepared as an acetone dried powder. The reconstitution of the mycelium was effected at 4°C and the preparations stored at the same temperature for the duration of the experiment.

3.4.i Sulfhydryl reductants

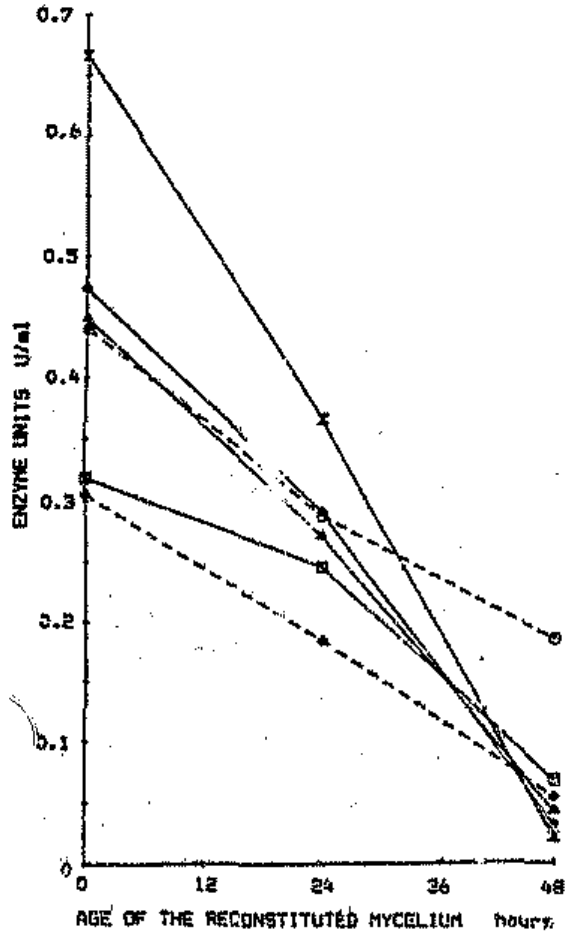
The concentration of the sulfhydryl reductant, DTE, was varied from 1 mM to 5 mM. β-mercaptoethanol at concentrations of 10 mM and 20 mM was also included in this study. Otherwise the buffer was kept constant at 10 mM Tris HCl, pH 7.5, 1 mM EDTA. The results are summarised in table 12 and figure 13. The most important feature of the results is that a 60 % loss was still observed after 48 hours. The increased DTE failed to reduce the loss of enzyme activity. Equally the alternative sulfhydryl reductant, 2-mercaptoethanol, while showing a slightly smaller loss of activity over 3 days, still lost sixty percent of its activity over this period. It is of interest to note that both the higher DTE and 2-mercaptoethanol concentrations (3 mM / 5 mM and 20 mM respectively) led to a more unstable reconstitution. One possible explanation of the greater instability of the enzymes at higher concentrations of sulfhydryl reductants is that the reductants themselves are interfering with the catalytic function of the enzymes. The addition of iodoacetamide, a sulfhydryl inhibitor, to assay solutions has been reported to inhibit FPP synthase (Holloway and Popjak, 1968, 1969). Notwithstanding this anomaly, it became clear from the results that the sulfhydryl reductants were not implicated in the destabilisation of the reconstituted mycelium and at the concentrations used in the reconstitution and purification buffers (1 mM, 2 mM and 3 mM) were sufficient for their task. Thus the study turned to divalent cations.

TABLE 12 : The effect of sulfhydryl reductants in the reconstitution buffer on the stability of the enzymes of the crude preparation.

	Sulfhydryl reductant	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	Day 1 % Loss	Day 2 U/ml	Day 2 % Loss
1	1 mM Dithioerythritol	0.22	0.47	0.29	38	0.03	94
2	2 mM Dithioerythritol	0.20	0.32	0.25	22	0.07	78
3	3 mM Dithioerythritol	0.20	0.45	0.27	40	0.04	91
4	5 mM Dithioerythritol	0.29	0.67	0.36	46	0.03	96
5	10 mM β-Mercaptoethanol	0.24	0.44	0.29	34	0.18	59
6	20 mM β-Mercaptoethanol	0.24	0.30	0.18	40	0.06	80

The basic buffer contained 10 mM Tris HCl (final - pH 8.0), 1 mM EDTA (final).

FIGURE 13: The stability of the enzymes of the crude preparation over a two day period in buffers containing different concentrations of sulfhydryl reductants. The respective concentrations are 1 mM DTE (○,——), 2 mM DTE (□,——), 3 mM DTE (△,——), 5 mM DTE (×,——), 10 mM β-MEtOH (□,--) and 20 mM β-MEtOH (○,--).



3.4.2 Divalent cations

The catalytic activities of the fungal forms of both FPP synthase and isomerase show a requirement for divalent cations (McGrath et al, 1977). FPP synthase had a preference for magnesium at an optimal concentration in excess of 5 mM. Concentrations of more than 1 mM Mn^{2+} inhibited FPP synthase. They also reported that β-CPA synthase showed no requirement for either divalent cation. Manganese was in fact found to have a inhibitory effect on β-CPA synthase while magnesium had little effect. For this experiment buffers were supplemented with various concentrations of both divalent cations to see whether the divalent cations had any effect on the stability of the enzymes themselves over and above their function as cofactors for catalytic activity.

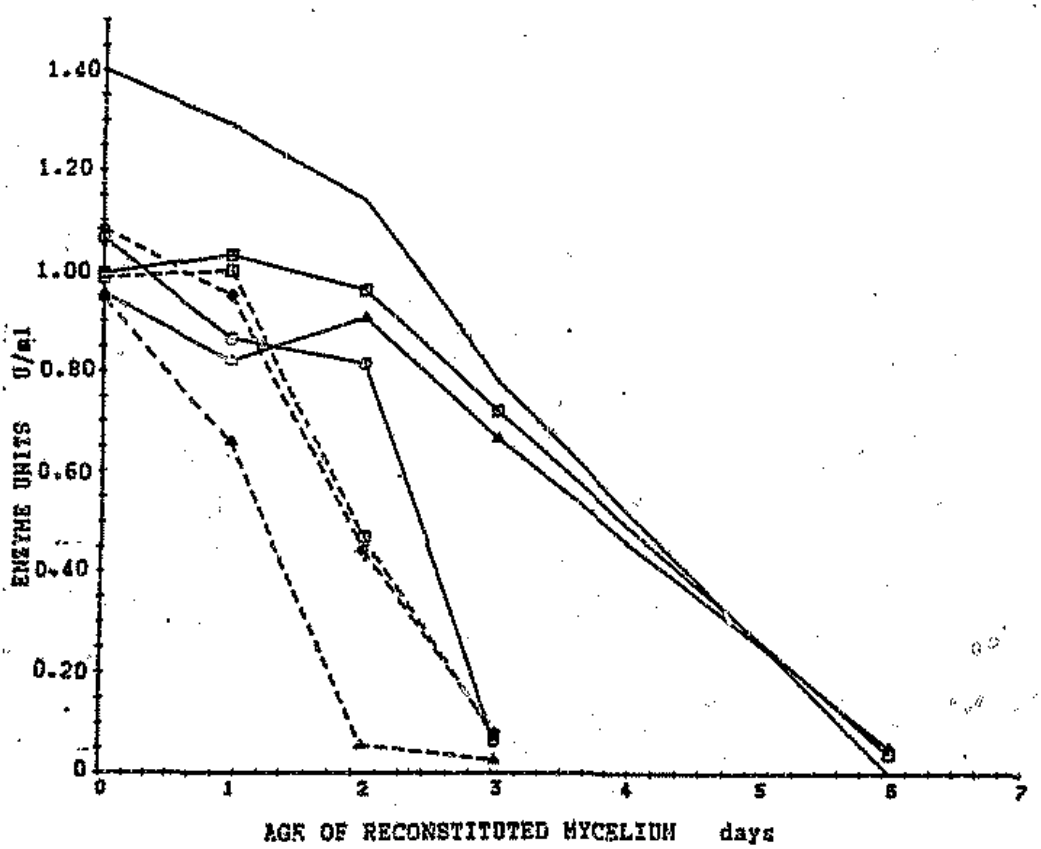
For each test the reconstitution buffer (10 mM Tris HCl, pH 7.5, 2 mM DTE and 1 mM EDTA) was supplemented with magnesium sulphate (1 mM, 2 mM or 5 mM), manganese sulphate (1 mM or 2 mM) or both (1 mM of each). The control was not supplemented with either cation. The results are shown in table 13 and figure 14. Supplemental manganese had a destabilising effect on the reconstituted mycelium and buffers supplemented with manganese were thus disregarded. The combination of magnesium and manganese (1 mM of each) showed a similar stability profile to that of 1 mM manganese and was also disregarded based on the destabilisation by the manganese. The lower concentrations of magnesium (1 mM and 2 mM) however showed a stabilising effect on the reconstituted mycelium when compared to the control. The 5 mM magnesium-supplemented buffer showed less stability than the control. The effective concentration of the supplemental cations was less than the 1 mM, 2 mM or 5 mM added due to the chelation of the ions by the 1 mM EDTA present in the buffer. Providing that the concentrations of any other ions present were negligible 1 mM EDTA would be able to bind around 1 mM magnesium. Thus for buffers of the most stable reconstituted mycelium, 1 mM and 2 mM magnesium, there would in fact be little free magnesium and no free EDTA present in the buffer. The reconstituted mycelium in the 5 mM magnesium buffer would have extra magnesium and the control would have

TABLE 13 : The effect of divalent cations in the reconstitution buffer on the stability of the enzymes of the crude preparation.

	Divalent cation	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	% Loss	Day 2 U/ml	% Loss	Day 3 U/ml	% Loss	Day 6 U/ml	% Loss
1	No divalent cation	0.060	1.40	1.29	8	1.14	19	0.78	44	0.02	99
2	1 mM Magnesium Sulphate	0.044	1.00	1.03	0	0.96	4	0.72	28	0.04	96
3	2 mM Magnesium Sulphate	0.042	0.95	0.82	14	0.91	4	0.66	31	0.05	94
4	5 mM Magnesium Sulphate	0.044	1.07	0.87	19	0.82	23	0.07	93		
5	1 mM Manganese Sulphate	0.046	0.99	1.00	0	0.47	52	0.08	92		
6	2 mM Manganese Sulphate	0.044	0.94	0.66	30	0.06	94	0.03	97		
7	1 mM of each cation	0.054	1.08	0.95	12	0.44	59	0.08	93		

The basic buffer contained 10 mM Tris HCl (pH 8.0), 2 mM DTE, 1 mM EDTA.
All concentrations are final.

FIGURE 14 : The stability of the enzymes of the crude preparation in a basic 10 mM Tris HCl, pH 8.0, 2 mM DTE, 1 mM EDTA buffer supplemented with 1 mM $MgSO_4$ (□,—), 2 mM $MgSO_4$ (△,—), 5 mM $MgSO_4$ (○,—), 1 mM $MnSO_4$ (□,--), 2 mM $MnSO_4$ (△,--) and 1 mM each of $MgSO_4$ & $MnSO_4$ (○,--). The control (—) was not supplemented with any cation.



free EDTA - both were less stable than the 1 mM and 2 mM magnesium buffers. As a result it was decided to investigate the effect of EDTA as a chelating agent on the stability of the reconstituted mycelium.

3.4.3 Chelating agents

The effect of EDTA at concentrations of 0 mM, 1 mM and 2 mM was investigated. Otherwise the reconstitution buffer remained as 10 mM Tris HCl, pH 7.5, 2 mM DTE. No divalent cations were added. The results are summarised in table 14 and figure 15. The investigation was not carried on beyond the second day when it became clear that the addition of EDTA to the reconstitution buffer was a major destabilizing factor. From the table it can be seen that the enzymes of the reconstituted mycelium preparations where EDTA was present lost more than 80 % of their activity in the first 24 hours. The reconstituted preparation using 0 mM EDTA in the buffer had two thirds of the enzyme activity still remaining after two days of reconstitution. It was concluded that the presence of EDTA in the reconstitution buffer was a major destabilising influence on the reconstituted mycelium.

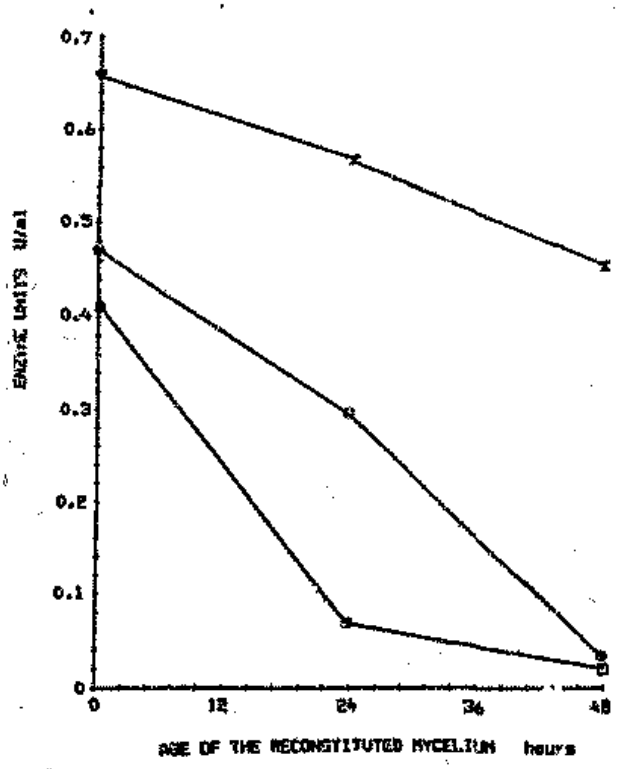
Hereafter EDTA was only included in the reconstitution buffer and an equal amount of $MgSO_4$ was always added as well. The rationale behind this is the operation of the Mg^{2+}-EDTA buffer that would chelate other ions such as Fe^{3+} and Zn^{2+} that may have been detrimental to activity of the enzymes. Although, after the separation of the mycelium's from the culture medium in a buchner funnel, the mycelium's were washed in buffer, the Mg^{2+}-EDTA buffer was still included in case of any remaining ions that may have had a detrimental effect on the stability or activity of the enzymes. For buffers used in the actual purification of the enzymes both $MgSO_4$ and EDTA were omitted except for as mentioned the reconstitution buffer.

TABLE 14 : The effect of chelating agents in the reconstitution buffer on the stability of the enzymes of the crude preparation.

	Chelating agent	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	Day 1 % Loss	Day 2 U/ml	Day 2 % Loss
1	0 mM EDTA	0.30	0.66	0.54	39	0.45	32
2	1 mM EDTA	0.22	0.47	0.29	86	0.03	94
3	2 mM EDTA	0.23	0.41	0.07	84	0.02	75

The basic buffer contained 10 mM Tris HCl (final - pH 8.0), 2 mM DTE (final).

FIGURE 15 : The stability of the enzymes of the crude preparation in a 10 mM Tris HCl, pH 8.0, 2 mM DTE buffer containing 0 mM EDTA (x), 1 mM EDTA (o) and 2 mM EDTA (□).



3.4.4 Glycerol

The effect of supplementing the reconstitution buffer with glycerol was also examined. A preliminary experiment showed that supplementing the reconstitution buffer with 10 % glycerol showed significant stabilisation of the reconstituted mycelium. Further studies on the effect of lycerol were carried out; first with concentrations of 10 %, 15 % and 20 % and then with concentrations of 0 %, 2 %, 4 %, 6 %, 8 % and 10 %. The standard 10 mM Tris HCl, pH 7.5, 2 mM DTE buffer was used as the basis for both studies.

Firstly buffers that were supplemented with 10 %, 15 % and 20 % glycerol were examined. The results are summarized in table 15 and figure 16. From the units remaining on day 6 it can be seen that increasing the glycerol concentration above 10 % had no measurable advantage apropos the stability of the reconstituted mycelium. Furthermore the increased concentrations did markedly increase the viscosity of the buffers at 4°C.

The reconstituted mycelium which was reconstituted in 10 % glycerol overnight had a third more protein and two thirds more enzyme activity than the corresponding buffer which had been reconstituted for only 2 hours. One possible explanation for this observation is that the enzymes while being soluble might have been weakly associated with the cell membrane. Longer reconstitution times would result in more dissociation from the cell membrane and less of the enzymes being lost in the pellet on centrifugation. The 20 % glycerol / 1 mM $MgSO_4$ reconstitution preparation yielded less protein than that of the 20 % glycerol buffer preparation and hence total enzyme units but was in all other respects similar to the latter.

One of the problem of supplementing the reconstitution buffer with glycerol was that the increased viscosity of the buffer caused longer processing times and poorer resolution on the gel filtration column. Since increasing the concentration of glycerol above 10 % had no added advantage as far as stability was concerned concentrations of 0 %, 2 %, 4 %, 6 %, 8 % and 10 % (v/v) were examined. The other elements of the buffers were

3.4.4 Glycerol

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TABLE 15 : The effect of glycerol supplemented reconstitution buffers on the stability of the enzymes of the crude preparation - 1.

Percentage concentration	Protein mg/ml	Day 0 Units	Day 1 Units	% Loss	Day 2 Units	% Loss	Day 3 Units	% Loss	Day 6 Units	% Loss
10% glycerol	0.53	1.26	0.97	23	1.14	10	1.23	2	1.06	16
15% glycerol	0.54	1.14	0.92	20	0.97	15	1.03	10	0.94	18
20% glycerol	0.60	1.53	1.32	13	1.60	0	1.47	4	1.09	29
20% glycerol/ 1 mM magnesium	0.29	0.76	0.62	19	0.73	5	0.62	18	0.54	30
10% glycerol overnight	0.71	2.09	1.61	23	1.99	5	1.85	11	1.54	26

The basic buffer contained 10 mM Tris HCl (pH 8.0), 2 mM DTE (both final concentrations) All cultures were reconstituted for 2 hours with the exception of the 10 % glycerol that is specified as overnight. No EDTA whatsoever was added to the reconstitution buffer.

FIGURE 16 : The stability of the enzymes of the crude preparation over a six day period in buffers supplemented with varied concentrations of glycerol - 1. These concentrations are 10% (•), 15% (▫), 20% (▪) and 1 mM $MgSO_4$ / 20% glycerol (x). Also included is 10% overnight reconstitution (—).

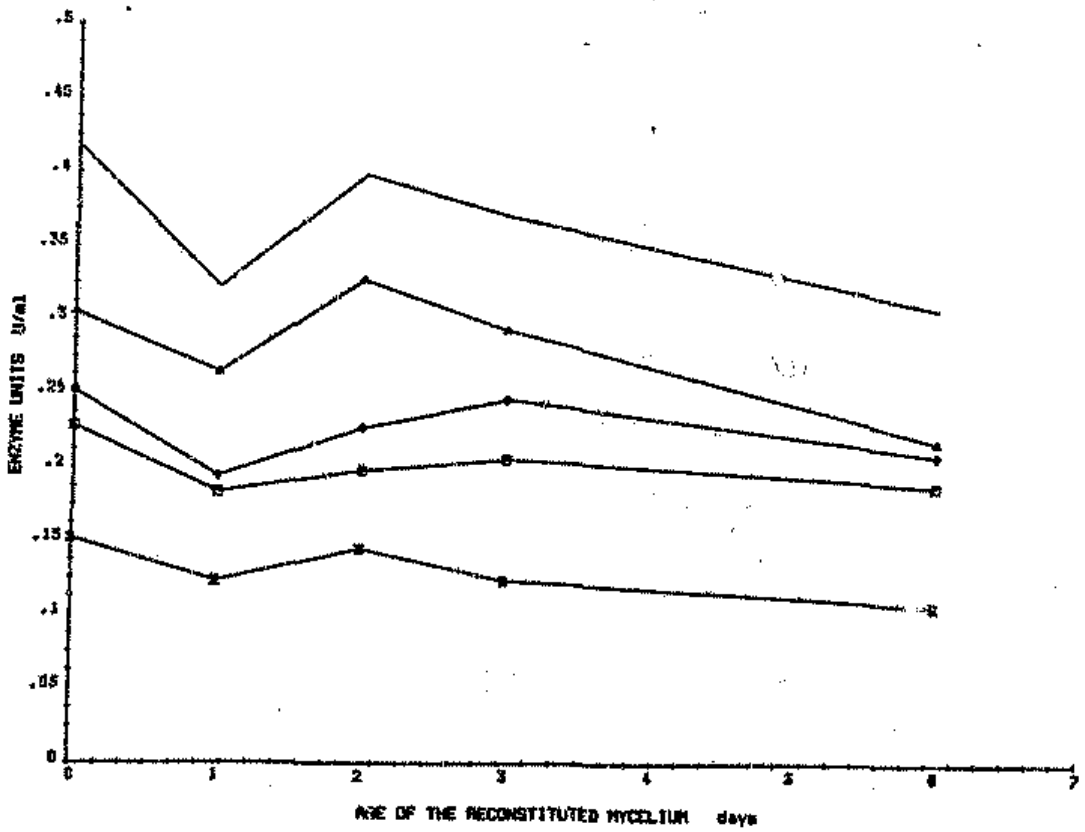
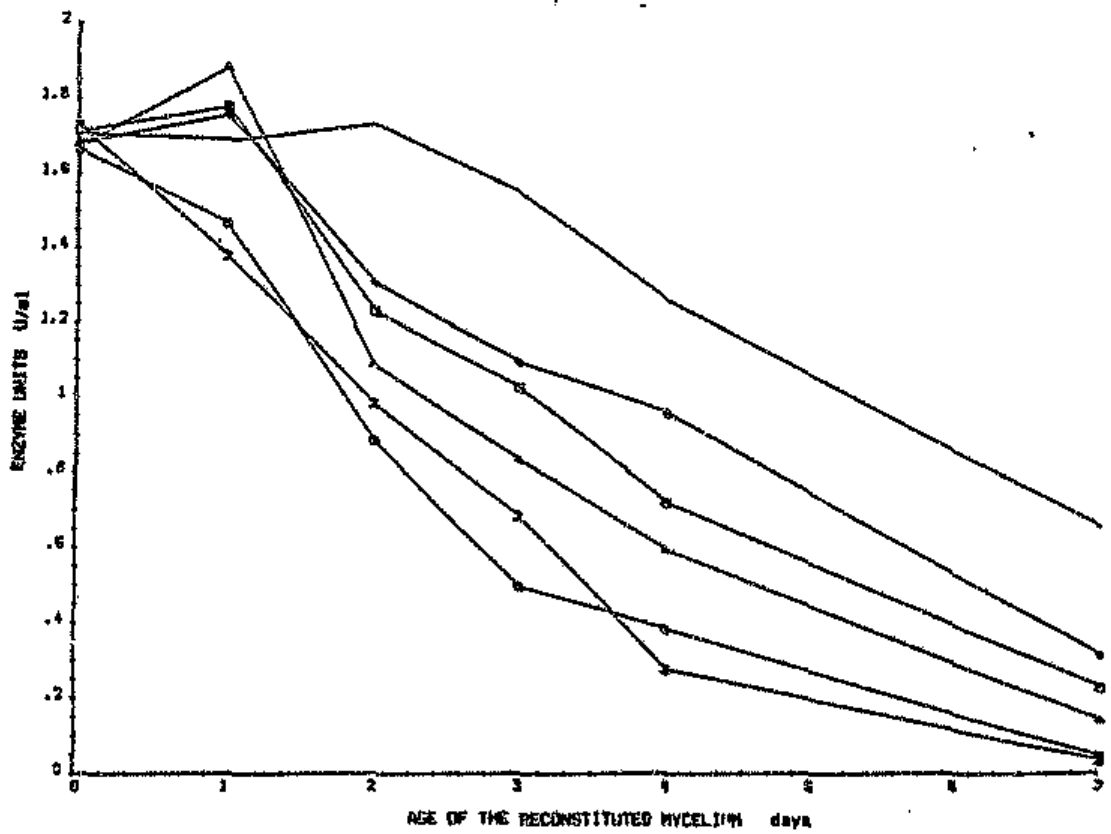


TABLE 16 : The effect of glycerol supplemented reconstitution buffers on the stability of the enzymes of the crude preparation - 2.

Percentage concentration	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	% Loss	Day 2 U/ml	% Loss	Day 3 U/ml	% Loss	Day 6 U/ml	% Loss
0% glycerol	1.29	1.663	0.732	56	0.884	47	0.497	70	0.381	77
2% glycerol	1.34	1.726	0.689	60	0.981	43	0.683	60	0.274	84
4% glycerol	1.37	1.676	0.940	44	1.085	35	0.830	50	0.590	65
6% glycerol	1.34	1.715	0.868	48	1.229	28	1.024	40	0.718	58
8% glycerol	1.25	1.683	0.877	46	1.305	22	1.091	35	0.955	43
10% glycerol	1.23	1.707	0.844	51	1.728	0	1.545	9	1.255	26

The basic buffer contained 10 mM Tris HCl (pH 7.9), 2 mM DTE (both final concentrations)
All cultures were reconstituted overnight at 4°C.
No EDTA was added to the reconstitution buffer.

FIGURE 17 : The stability of the enzymes of the crude preparation over a six day period in buffers supplemented with different concentrations of glycerol - 2. These concentrations are 0% (○), 2% (×), 4% (△), 6% (□), 8% (◇) and 10% (—) glycerol (final volume).



10 mM Tris HCl, pH 7.5 and 2 mM DTE. The results are shown below in table 16 and figure 17. The results show that for the 10 % glycerol buffer the reconstituted mycelium was completely stabilised for the first two days, after which the enzymes gradually lost activity. The graphical relationship (figure 17) between the concentration of the glycerol buffer and the enzyme units suggests that the initial stabilisation of the reconstituted mycelium by these six glycerol reconstitution buffers is proportional. After the initial period they lose activity at roughly an equal rate.

3.4.5 Buffer pH

Although proteins are known to be more stable at pH's of around 8.0 it was decided to examine the effect of pH on the reconstituted mycelium's stability because the reported pI's were around pH 5 and isoelectric focusing was planned as the last step in the purification process. The buffer pH's that were studied were 8.0, 7.5, 7.0, 6.5 and 6.0. The buffer consisted of 10 mM Tris HCl, 2 mM DTE and 10 % (v/v) glycerol and was adjusted to the required pH with maleic acid. Maleic acid was used in place of HCl because at pH's of less than 7.0 Tris HCl has no buffering capacity. The results are shown in table 17 and figure 18.

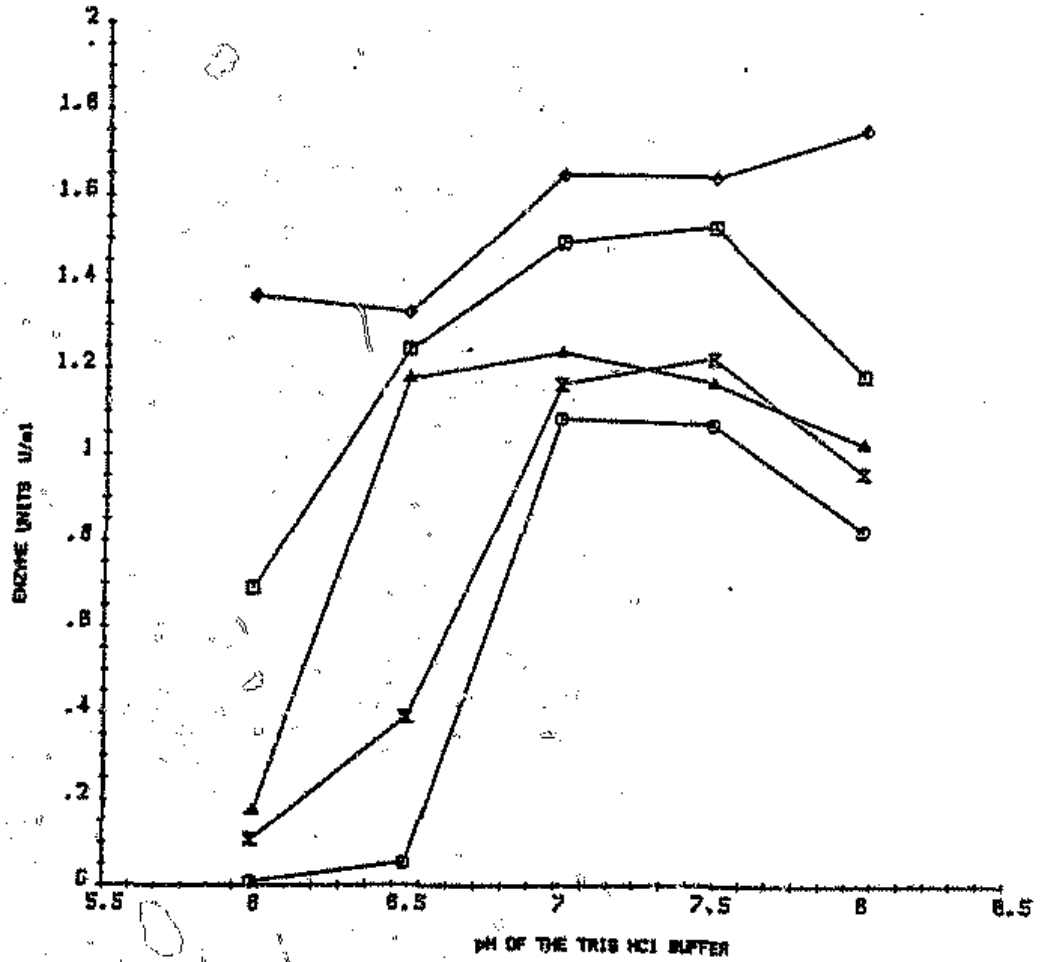
At pH 6.0 the reconstituted mycelium showed a 49 % loss over the first 24 hours and a total loss of 87 % by the end of the second day. The pH 6.5 buffer showed a sharp drop in activity on the fourth day. At pH 8.0 the reconstituted mycelium showed an initial drop in activity that flattened out and after 7 days 47 % of the activity remained. Otherwise the remaining two buffers, at pH's 7.5 and 7.0, showed a similar stability profile over the required three day period. Following on from this experiment pH 7.5 was used for the purification of the enzymes.

TABLE 17 : The effect of pH of the reconstitution buffer on the stability of the enzymes of the crude preparation.

	Buffer pH	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	% Loss	Day 2 U/ml	% Loss	Day 3 U/ml	% Loss	Day 7 U/ml	% Loss
1	8.0	0.93	1.753	1.183	33	1.024	42	0.957	45	0.825	53
2	7.5	0.84	1.644	1.528	7	1.166	29	1.223	26	1.031	37
3	7.0	0.77	1.648	1.492	9	1.236	25	1.163	29	1.037	95
4	6.5	0.66	1.328	1.244	6	1.176	11	0.391	71	0.033	96
5	6.0	0.41	1.366	0.691	49	0.174	87	0.108	92	0.009	99

The basic buffer contained 10 mM Tris, 2 mM DTE and 10 % glycerol.
The buffers were adjusted to the desired pH's with HCl.
All cultures were reconstituted overnight at 4°C.

FIGURE 18 : The stability of the enzymes of the crude preparation in a 10 mM Tris HCl, 2 mM DTE, 10% glycerol buffer at pH's of 6.0, 6.5, 7.0, 7.5 and 8.0. Assays were done 24 hourly and are represented thus ; day 0 (◇), day 1 (□), day 2 (△), day 3 (✕) and day 7 (○).



3.4.6 Buffer ionic strength

During initial ion exchange experiments it was observed that the fungal enzymes tended to be more stable at the higher ionic strengths than the $\mu = 0.01$ (10 mM Tris HCl) that had been previously used for all general buffers. These higher ionic strengths were a result of the buffers used to generate the linear gradient (100 mM to 400 mM Tris HCl). Consequently a range of Tris HCl concentrations were examined with respect to the activity of the reconstituted mycelium's enzymes. These concentrations were 10 mM, 20 mM 50 mM, 100 mM and 200 mM. Each buffer contained 2 mM DTE but since in the ion exchange buffer glycerol was not present it was decided to omit glycerol from the buffer for this experiment. The results are reflected in table 18 and figure 19.

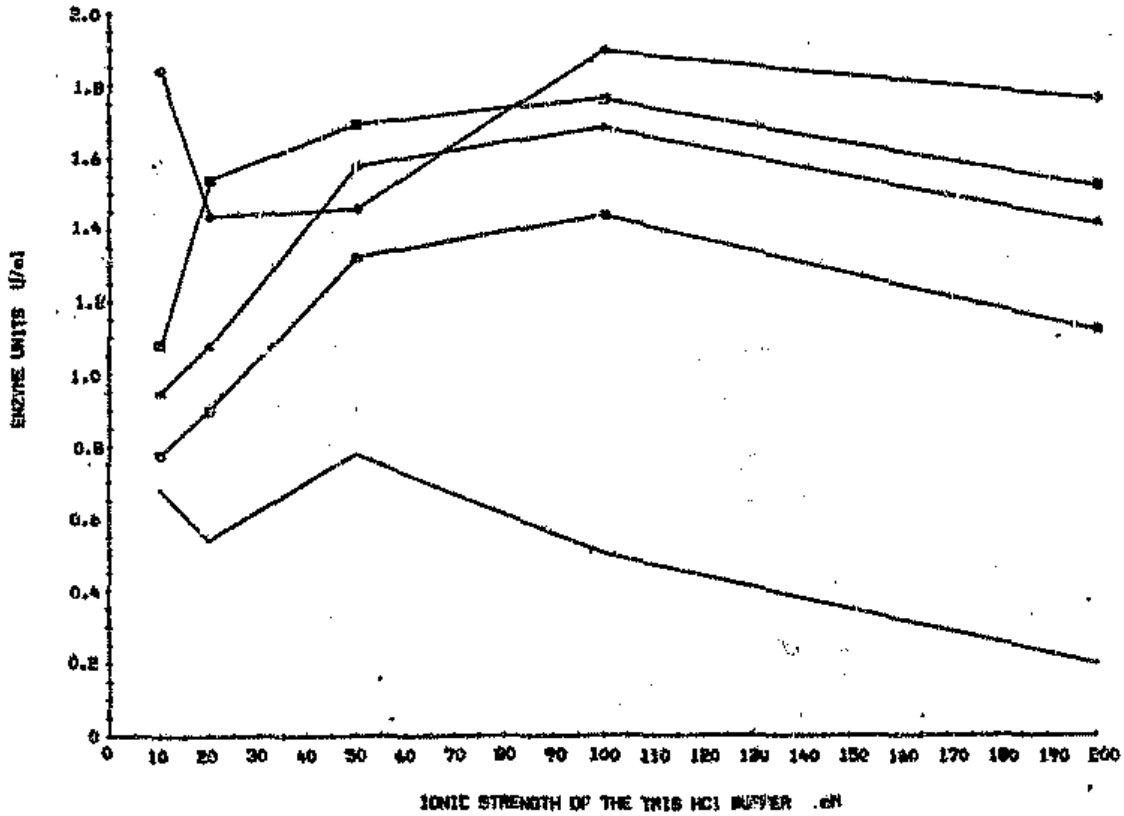
From the results it can be seen that 50 mM appeared to offer the optimal concentration of Tris HCl. It is interesting to note that while the enzymes in the lower two buffer concentrations initially lost activity rapidly, the rate of activity loss flattened out considerably and the losses from days three to seven were 40 % and 30 % for the 20 mM and 10 mM buffers respectively. On the other hand the 200 mM and 100 mM Tris HCl preparations showed stability for the first 2 days before losing activity at a far greater rate than the rest over the remaining five days of the experiment. From this a buffer concentration of 50 mM Tris HCl was selected for the purification of the enzymes.

TABLE 18 : The effect of the ionic strength of the reconstitution buffer on the stability of the enzymes of the crude preparation.

	Buffer	Protein mg/ml	Day 0 U/ml	Day 1 U/ml	% Loss	Day 2 U/ml	% Loss	Day 3 U/ml	% Loss	Day 7 U/ml	% Loss
1	200 mM Tris HCl	0.86	1.936	1.671	14	1.556	20	1.163	40	1.232	36
2	100 mM Tris HCl	0.98	2.094	1.936	7	1.849	11	1.789	14	1.581	24
3	50 mM Tris HCl	0.91	1.601	1.858	0	1.731	0	1.586	0	1.454	9
4	20 mM Tris HCl	0.85	1.579	1.691	0	1.183	25	0.978	38	0.988	37
5	10 mM Tris HCl	0.84	2.027	1.191	41	1.041	49	1.035	49	0.854	58

The basic buffer contained varying concentrations of Tris.
Each buffer was adjusted to a pH of 8.0 with HCl.
Each buffer was supplemented with 2 mM DTE.
None of the buffers were supplemented with any glycerol.
All cultures were reconstituted overnight at 4°C.

FIGURE 19 : The stability of the enzyme of the crude preparation in Tris HCl buffer, pH 7.5, at concentrations of 10 mM, 20 mM, 50 mM, 100 mM and 200 mM. Assays were done 24 hourly and are represented thus : day 0 (◇), day 1 (□), day 2 (△), day 3 (○) and day 7 (—).



CHAPTER FOUR

PURIFICATION OF THE FVIIIA: ENZYMES

The instability of the fungal enzymes led to the purification method that had been developed for the porcine liver being modified. These modifications were to satisfy two criteria - firstly to reduce the harshness with which the enzyme was handled and secondly to minimize the actual time spent on purifying the enzymes. These two criteria had to be met without compromising the resolution of the purification. Glycerol, the major stabilizing element of the reconstitution buffer, was thus included only for the initial reconstitution of the mycelium. It was also included at the end for storage once the various purification steps had all been performed. The reason for this was that, although glycerol was found to stabilise the enzymes, glycerol had an adverse affect on the resolution of the enzymes on the gel filtration and ion exchange steps.

Furthermore in all purification steps with the exception of the isoelectric focusing the pH of the buffer was kept between 7.5 and 7.9 and the buffer strength kept at 50 mM Tris HCl. Similarly the concentration of the sulfhydryl reductant, DTE, was kept at 2 mM and only the reconstitution buffer was supplemented with EDTA and then it was supplemented by an equal amount of magnesium sulphate in line with the observation of 3.4.2 above. The time required to effect the crude fraction, gel filtration, ion exchange and isoelectric focusing steps was reduced to 72 hours. The problem of instability of the enzymes was further complicated by the large losses found on the isoelectric focusing steps. This will be discussed later (4.4).

The poor stability of fungal enzymes compared to enzymes of vertebrate origin is well documented (Eberhardt and Rilling 1975, Takahashi et al 1978, Cress et al 1981). A major contributing factor for this instability must be the harsh methods needed to rupture the mycelial wall. Coupled with the lower relative amount of soluble protein present in a fungal mycelium in comparison to a vertebrate cell, the harshness of

the method of mycelial rupture meant that an inordinate amount of effort must be spent to optimize the amount of unimpaired protein extracted from the mycelium. The use of homogenization for the fungus was attempted (3.3.2) failed to produce anything near the amount of protein that the porcine liver purification produced (0.11 mg/ml against 10.9 mg/ml). Thus it was necessary to adopt a new method of mycelium rupture. The development of a satisfactory method of mycelial rupture is discussed above (3.3.2). Freeze drying was eventually adopted as it was the least harsh of the viable mycelial rupture methods available.

Freeze dried stocks of ruptured mycelium were prepared and stored for more than 12 months at -30°C without any significant loss of activity. Two stocks of freeze dried mycelium were prepared using the aspartate-based culture media developed in 3.3.4 above. The secondary innocula for these two stocks were grown for 36 and 48 hours. For each purification a 1:1 ratio (w/w) of these two stocks were used for the reconstituted mycelium. The reason for this is that the 36 hour stock had slightly more FPP synthase than β-CPA synthase while the 48 hour stock had very little FPP synthase and a substantial amount of β-CPA synthase. By combining the two stocks a reasonable yield of these two enzymes could be achieved from the reconstituted mycelium. Both stocks were on occasion purified separately and no untoward effects were evident from the combination of the two stocks.

4.1 Crude fractionation

Secondary innocula, grown in culture medium with a sodium aspartate concentration of 10 g/l as the nitrogen source and a glucose concentration of 80 g/l, were grown for a period of either 36 hours or 48 hours. The mycelia were washed with 50 mM Tris HCl, pH 7.5, 2 mM DTE, 1 mM each of EDTA and $MgSO_4$, 10% (v/v) glycerol buffer before the mycelial walls were ruptured by freeze drying. The stock powder was stored at -30°C for long periods and used as required. No undue interference from

the trace elements of the culture medium were observed to affect the reconstituted mycelium or the purification. Reconstitution of the mycelium for the enzyme purification was overnight in 50 mM Tris HCl, pH 7.5, 2 mM DTE, 1 mM each of EDTA and $MgSO_4$, 10% (v/v) glycerol) buffer.

The reconstituted mycelium was centrifuged and filtered. After samples had been taken for enzyme and protein assays this fraction, the crude preparation, was concentrated on an Amicon concentrator. The protein concentration increased eight fold (1.04 mg/ml to 8.16 mg/ml - table 19).

4.2 Gel filtration

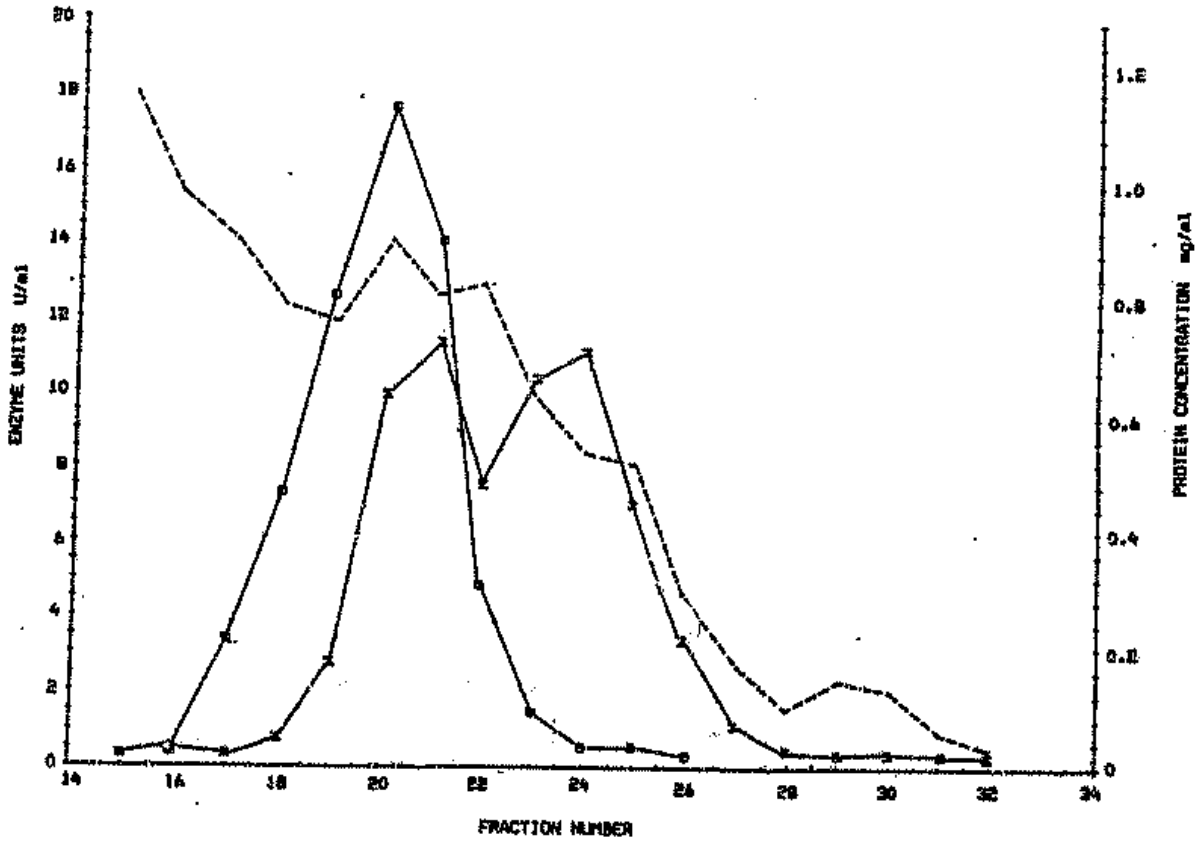
The concentrate of the crude preparation (4.5.1) was loaded at a rate of 18 ml/hour onto a AcA 44 gel filtration column which had previously been equilibrated with 50 mM Tris HCl, pH 7.5, 2 mM DTE. Blue dextran, which was used as the front marker, was loaded before the concentrate. Myoglobin, the back marker, was loaded only after the concentrate had entered the bed of the gel filtration column. These steps were taken to avoid any possible interaction between the markers and the desired enzymes. The gel filtration column was run overnight. A typical elution profile is shown in figure 20. Fractions of 13 ml were collected. The synthases were found to elute from the column at much the same position, while the isomerase was eluted from the column after the synthases. Because the FPP synthase and isomerase assays use a common substrate, in figure 20 the first FPP synthase peak represents the FPP synthase while the second peak is actually the contaminating isomerase.

The fractions were assayed for protein and enzyme activity. Typical results are shown in table 19. Typically the purification factor for the gel filtration step was in the region of 1½ for the concentrate and 3 for the purification as a whole. However because of the somewhat unspecific nature of the results of the coupled assay the purification factor has

TABLE 19 : A typical protein purification profile of the enzymes of *Penicillium cyclopium*.

Fraction	Coupled assay	FPP synthase assay	A-CPA synthase assay
Filtrate			
Protein conc. mg/ml	1.04		
Enzyme units U/ml	6.38		
Specific act. U/mg	6.13		
Concentrate			
Protein conc. mg/ml	8.16		
Enzyme units U/ml	70.8		
Specific act. U/mg	8.68		
Purification factor	1.42		
Gel filtration			
Protein conc. mg/ml		0.81	0.90
Enzyme units U/ml		11.2	17.5
Specific act. U/mg		13.8	19.4
Purification factor		2.3	3.2
Ion exchange			
Protein conc mg/ml		0.37	0.58
Enzyme units U/ml		13.0	7.70
Specific act. U/mg		35.5	40.1
Purification factor		5.8	6.5
Isoelectric focusing			
Protein conc mg/ml		17.0	17.0
Enzyme units U/ml		0.67	0.70
Specific act. U/mg		39.4	41.2
Purification factor		6.4	6.7

FIGURE 20 : The elution profile of the fungal enzymes from an AcA 44 gel filtration column (80 cm × 2.6 cm) at a flow rate of 18 ml/hour. The eluent was assayed for FPP synthase (×) and β-CPA synthase (○) activity, and the protein concentration (--) determined.



meaning only in so far as comparison with other purification procedures is concerned. In other words, not knowing the relative distribution of FPP, GPP and β-CPA /α-CPA from the coupled assay, makes comparison of the results of enzyme specific assays (such for the gel filtration eluent) to the coupled assay only meaningful in comparison to other purifications of the enzymes and thus have no absolute meaning.

The fractions showing synthase activity were pooled and loaded onto the DE 52 ion exchange column. The synthase peaks and the peak from isomerase overlapped to a far greater extent than the porcine liver isomerase and FPP synthase had (see figure 8). Thus where the isomerase was a significant contaminant of a fraction and the amount of either of the synthases were not large, the fraction was discarded.

4.3 Ion exchange

Previous attempts to use ion exchange chromatography as a method of purifying the FPP and β-CPA synthases from Penicillium cyclopium had been unsuccessful (McGrath - personal communication). They found that the enzymes on elution from the ion exchange column lost all activity over a period of a few hours. As a result the use of an ion exchange column was first tested using crude preparations of reconstituted mycelium to see whether stable enzyme preparations could be obtained on elution from an ion exchange resin. The ion exchange resin that was selected was Whatman DE52. The use of this ion exchange resin for the separation of FPP synthase has been well documented (Barnard, 1984; Rilling, 1984; Satterwhite, 1984).

For the initial experiments with a crude preparation step wise elution using 300 mM KCl was used to elute the enzymes after they had been loaded and washed with a 50 mM Tris HCl, pH 7.5, 2 mM DTE buffer. The enzymes were assayed and found to be active. The enzymes assays were repeated five days later and around 70% of the enzyme activity was still present. Following on from this a gradient was attempted and found to elute the

enzymes within the range of 100 mM to 400 mM Tris HCl at pH 7.5. Further assays determined that the stability was in line with that found for the step wise elution with KCl. Having established that the enzymes were stable on elution from a DE 52 ion exchange resin, use was made of ion exchange as a means for purification.

The active fractions from the gel filtration column were loaded on to a equilibrated DE 52 ion exchange resin in a 50 mM Tris HCl, pH 7.5, 2 mM DTE buffer and washed with the same buffer until all unbound protein was eluted from resin. A 100 mM - 400 mM Tris HCl, pH 7.5, gradient was applied and the enzymes eluted. Fractions of 7 ml were collected and assayed for enzyme activity and protein. The order of elution from the ion exchange resin was firstly the β-CPA synthase and a small amount of isomerase, followed by the FPP synthase. There was signif cant overlap between the first two enzymes, with β-CPA synthase being marginally behind of isomerase. Typical results are shown in table 19 and an elution profile is to be seen in figure 21.

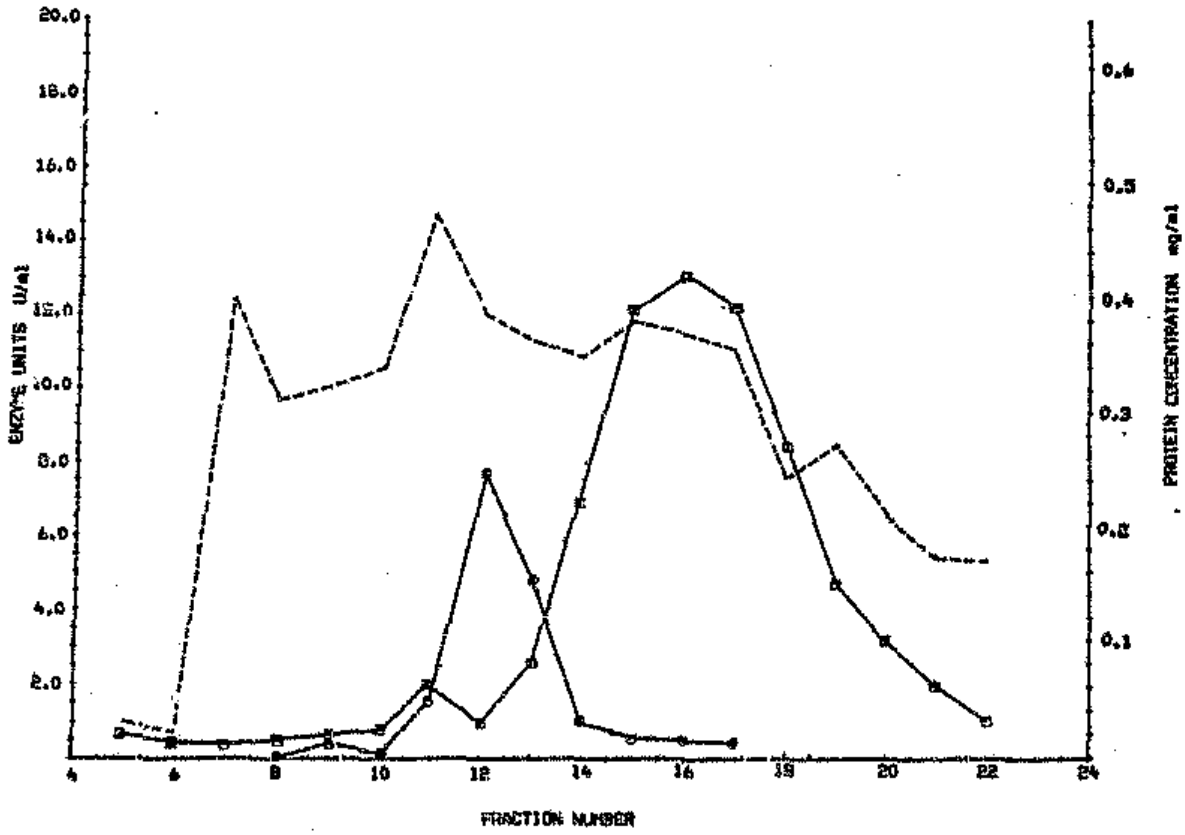
The active fractions of FPP synthase and β-CPA synthase were pooled and dialyzed overnight against a 2 mM Tris HCl pH 7.5, 2 mM DTE buffer in preparation for isoelectric focusing.

4.4 Isoelectric focusing

Initially great difficulty was experienced in getting the isoelectric focusing technique to function properly. The bed after various periods of focusing repeatedly showed all the characteristics of cathodic drift with the one exception that the drift was towards the anode. Cathodic drift is characterized by the movement of the liquid phase towards the cathode with a concomitant dehydration of the other end. In this case the build up of liquid was however at the anode.

The fault was eventually isolated to the electrofocusing gel. Replacement of the gel resulted in the pH gradient being formed in a linear manner but large protein losses were experienced during the

FIGURE 21 : The elution profile of the FPP and β-CPA synthases from a DE 52 cation exchange column (11 cm x 2.5 cm). The eluent was assayed for FPP synthase (□) and β-CPA synthase (○) activity, and the protein concentration (--) determined.



focusing itself. These losses were observed both when a crude preparation (as prepared in 4.1) and the pooled fractions from the ion exchange were focused. The addition of 1 % (v/v) of glycerol to the bed *together with the ampholytes* did not reduce the losses found on focusing. It was found that it was critical to determine the pH of the fractions and then to add the 1 ml of 100 mM Tris HCl, pH 7.5, 2 mM DTE buffer to the fractions as soon as possible after the power had been disconnected - an hour or two's delay after fractionating the bed meant a loss of more than 90 % of the enzyme activity. If however the pH's of the fractions were determined and then adjusted to within 0.3 pH units of pH 7.5 within a half an hour after the end of the focusing then there was sufficient residual activity to determine the pI's of the synthases and the isomerase. Typical isoelectric focusing profiles of focuses of the crude preparation and of the pooled fractions from the ion exchange column are shown in figure 22 and figure 23 respectively. The corresponding results are shown in tables 20 and 21 respectively. When the pH's of the fractions were determined special care was taken ensure that the pH meter was correctly calibrated.

From these figures and tables it can been seen that the pI's for the isomerase (4.5) and β-CPA synthase (5.3) compare favourably with the pI's reported by McGrath et al (1977). The pI of FPP synthase for both the crude preparation and the eluent from the ion exchange column were found to be 0.2 pH units lower than that reported by McGrath et al (1977) (4.9 as opposed to 5.1). For the FPP synthase assay of the eluent of the ion exchange column the enzyme activity peak was distributed between two adjacent fractions which had pH's of 4.83 and 4.98. The average, 4.91, was taken as the pI for that run.

Given the results of the experiments to determine the stability of the enzymes at various pH's and particularly the results from pH 6.0, the rapid loss of enzyme activity is not surprising since the isoelectric focusing is at pH's less than 6. The addition of 1 % glycerol had no effect on the loss of enzyme activity whatsoever. McGrath et al (1977)

TABLE 20 : Fractionation of a crude preparation of the fungal enzymes on an isoelectric focusing bed.

Fraction		
Load (coupled assay)		
Protein conc.	mg/ml	1.035
Enzyme units	U/ml	10.3
Specific act.	U/mg	9.95
FPP synthase		
Protein conc.	mg/ml	0.200
Enzyme units	U/ml	0.23
Specific act.	U/mg	1.15
fraction pH		4.91
β-CPA synthase		
Protein conc	mg/ml	0.118
Enzyme units	U/ml	0.41
Specific act.	U/mg	3.48
fraction pH		5.36
Isomerase		
Protein conc.	mg/ml	0.199
Enzyme units	U/ml	0.35
Specific act.	U/mg	1.76
fraction pH		4.44

FIGURE 22 : A profile of the gel after the isoelectric focusing of a crude preparation. The activity of the FPP (o) and β-LPS synthases (•) and the protein concentration (---) were determined. The pH gradient is shown thus (x).

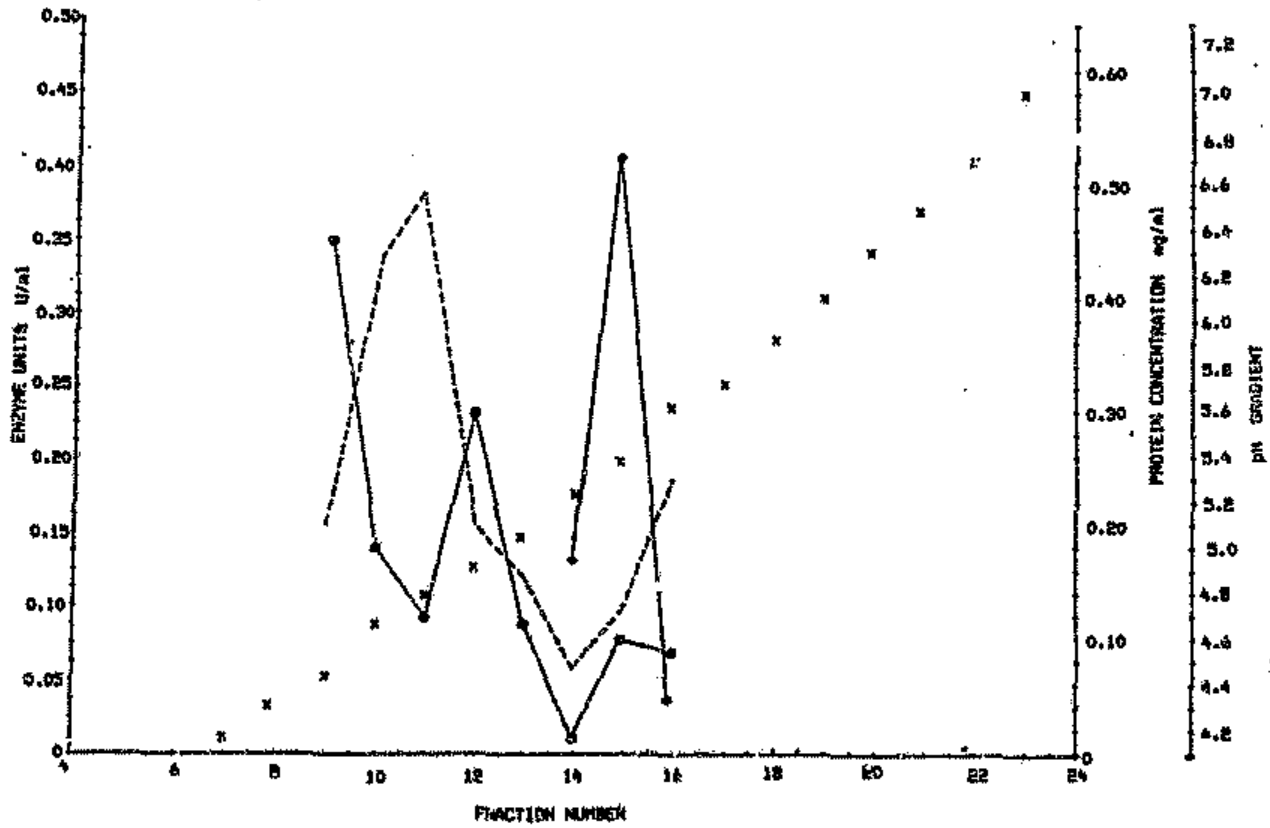
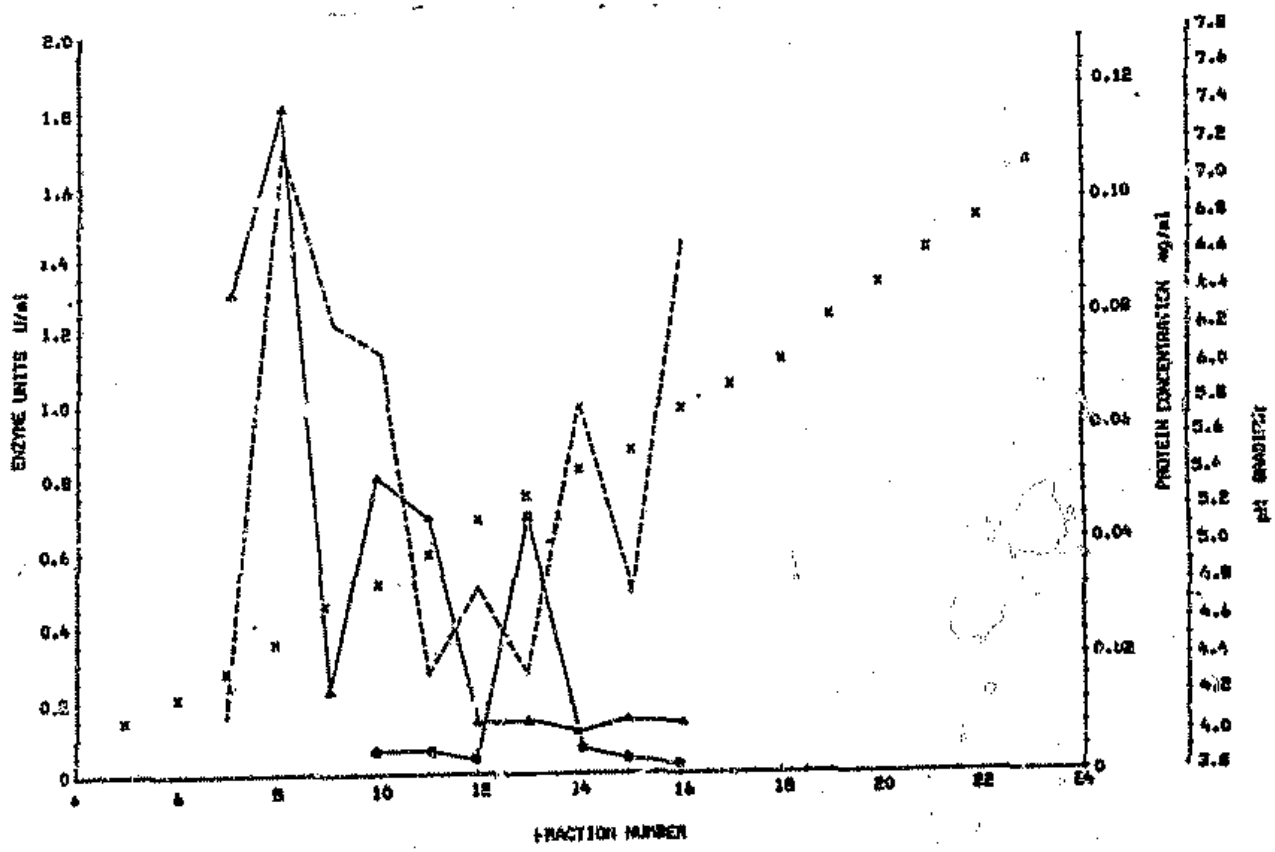


TABLE 21 : Fractionation of the pooled eluent of an ion exchange column on an isoelectric focusing bed.

Fraction		
Load (coupled assay)		
protein conc.	µg/ml	260
enzyme units	U/ml	2.94
specific act.	U/mg	11.3
FPP synthase		
protein conc.	µg/ml	17.0
enzyme units	U/ml	0.67
specific act.	U/mg	39.4
fraction pH		4.91
GGPA synthase		
protein conc	µg/ml	17.0
enzyme units	U/ml	0.70
specific act.	U/mg	41.2
fraction pH		5.27
Isomerase		
protein conc.	µg/ml	110
enzyme units	U/ml	0.35
specific act.	U/mg	1.76
fraction pH		4.50

FIGURE 23 : A profile of the gel after the isoelectric focusing of the pooled eluent of the ion exchange column. The activity of the FPP (▲) and β-CPA synthases (○) and the protein concentration (—) were determined. The pH gradient is shown thus (x).



also reported that their preparation from ammonium sulphate fractionation lost all activity during isoelectric focusing.

4.5.5 Polyacrylamide gel electrophoresis

Two types of polyacrylamide gels were run. The first contained samples of the active fractions for the eluents of the gel filtration and ion exchange chromatography steps and the second contained samples from the range spanning the bands on the isoelectric focusing where enzyme activity had been detected. BSA was run on all gels for comparison purposes. The gels were mostly run overnight at 25-35 volts.

For the first type of gel, the crude or concentrate (one or the other was used), together with the active fractions were run with BSA as the standard. The major bands observed in the crude preparation or the concentrate sample were did not correspond to the bands obtained from the active fractions of the gel filtration column. Problems were experienced with the detection of the bands from the ion exchange column. The length of staining was varied and most gels were stained for a full two hours but the detection of active bands for the ion exchange fractions was still problematic and in many cases no bands could be detected even though the fractions had assayed positive.

The problems with the detection of the bands from the ion exchange coupled with the fact that the protein concentrations of the fractions from the isoelectric focusing had lower protein concentrations than those of the ion exchange led to only the active fractions from the focusing of the crude preparations being run on polyacrylamide gel electrophoresis gels. Only two of the samples run on PAGE from the isoelectric focused fractions had protein bands that stained. Those were both in the region of the primary transferase. The one sample had a single band that had run a similar distance to BSA while the other sample had three bands that had barely moved from the loading wells. No trace of protein was ever observed in the pI regions of the β-CPA synthase or the isomerase.

Because of these problems that were experienced with the detection of protein on the PAGE gel no conclusion can be made from the use of polyacrylamide gel electrophoresis as to the degree of purity that was achieved other than the purification factors reported elsewhere.

4.5.6 Molecular weight determination

The excellent separation and reproducibility of the UltroGel AcA 44 gel filtration column led to it being used to estimate the molecular weights of the synthases. This gel has a fractionation range of 10 000 - 130 000 and a total exclusion of 200 000. McGrath et al (1977) reported the molecular weight of FPP synthase as 64 000 and β-CPA synthase as 96 000. Use was made of alcohol dehydrogenase (MW 150 000), BSA (MW 68 000), egg albumin (MW 45 000) and carbonic anhydrase (MW 29 000) to calibrate the AcA 44 gel filtration column. The slowest recommended flow rates (3 cm/hr - 15 ml/hr) were used and 3½ ml fractions were collected. Two determinations of the molecular weights of the synthases were done. The results are shown in table 22.

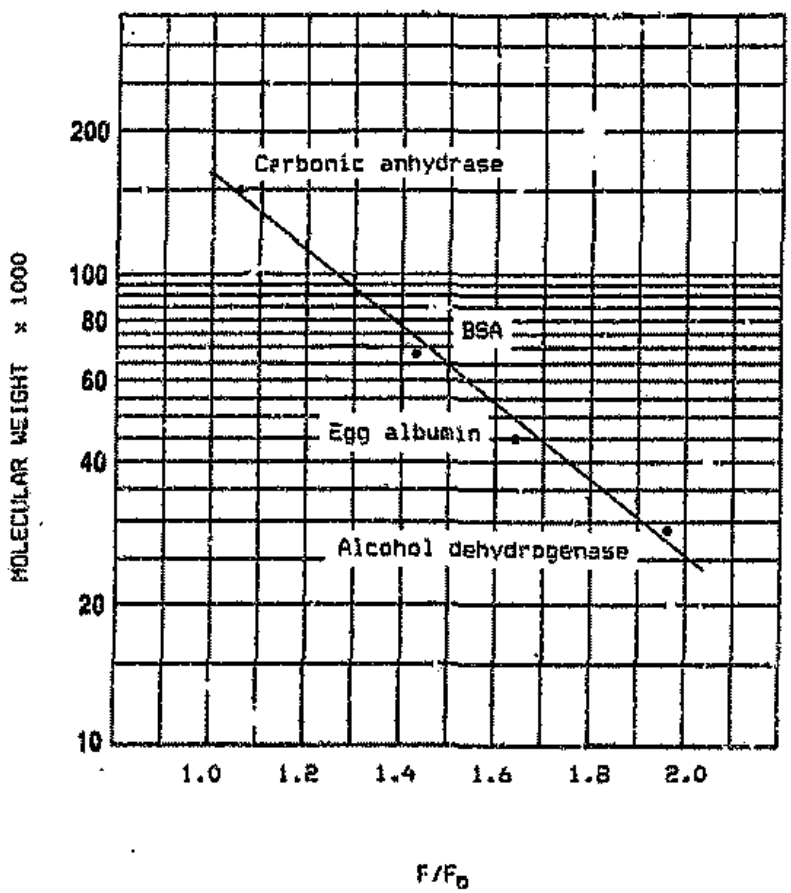
The molecular weight of β-CPA synthase was found to be 83 000 ±3900 and that of FPP synthase was found to be 77 600 ±3700. These results are in accordance with the general observation during purification that the the two synthases eluted in the same or adjacent fractions. McGrath et al (1977) reported different molecular weights (FPP synthase, 64 000 and β-CPA synthase, 96 000) but also reported varying molecular weights from growth to growth. Their β-CPA synthase molecular weight was found to be as low as 75 000 in the presence of magnesium ions. Whether or not this is an effect of the ice shear press method of mycelial rupture was not determined. They however reported values of between 50 000 (in the presence of manganese and magnesium) and 64 000 for FPP synthase. For the determination of the enzyme's molecular weights no magnesium was added to the buffer and so the lower molecular weight found for β-CPA synthase could not be attributed to this. Equally the FPP synthase determination,

TABLE 22 : A table of the estimation of the molecular weights of the FPP and β-CPA synthases.

Protein	Fraction number	Molecular weight	Log MW	F/Fo
Blue dextran	35	2 000 000		1.000
Alcohol dehydrogenase	37	150 000	5.18	1.057
BSA	50	68 000	4.83	1.429
Egg albumin	57	45 000	4.65	1.629
Carbonic anhydrase	69	29 000	4.46	1.971
FPP synthase	48	77 600 ±3700	4.89 ±.02	1.371
β-CPA synthase	47	83 000 ±3900	4.92 ±.02	1.343

The molecular weight determinations were done in duplicate.

FIGURE 20 : The calibration curve for the estimation of the molecular weights of FPP and δ-CPA synthases. The ratio of the fraction (F) over the void fraction (F_0) is plotted against the molecular weight in daltons.



for which a molecular weight of 77 600 was found, was in the same buffer as that of β-CPA synthase.

Molecular weight determination using the method of gel filtration is an approximate method at the best of times and the results must be accepted only on a provisional basis (Andrews, 1964). Furthermore it is desirable to select the fractionation range so as to minimize the flattening of the last protein peaks while enabling the proteins to separate sufficiently to enable their peak to be easily discernible. In other words the fractionation range of the gel filtration column should be selected so that the trade off between the clear separation of the protein peaks and the loss of resolution marked by excessive times on the column is minimised. A more satisfactory fractionation range for determining the molecular weight between 50 000 and 100 000 would be UltroGel AcA 34. Furthermore denaturing techniques such as SDS PAGE should also be used to determine a more definitive molecular weight for both of these synthases.

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CHAPTER FIVE

DISCUSSION AND CONCLUSION

This project began initially with the intention of purifying the FPP and β-CPA synthases and then investigating whether the two enzymes were homologous or not. However the instability of the enzymes and the concomitant need to achieve a certain minimal stability before the enzymes in question could be purified to a state where a comparison could be made caused the impetus to initially be directed at optimizing the stability of the enzymes. The purification of proteins for structural studies places an additional burden, when compared to purifying them for activity studies. The reason for this is that for the activity studies a high speed low resolution purification is used, while for structural studies a high resolution purification that is invariably much slower is required in order to remove all traces of contaminating protein and other materials. Where, as in this investigation, there was little enzyme activity to begin with and the only means of detecting the enzymes was by means of an enzyme activity assay, an unstable enzyme preparation constituted a major obstacle to the purification of enzymes for structural studies.

To this end the steps of the purification procedure had to be chosen so as to minimize the time taken for the purification and to minimize the harshness of the steps themselves. The most effective way appeared to be to choose purification steps that use different physical characteristics of the proteins for each step and which were designed to achieve the best resolution for that each step. In others words avoiding having duplicate purification steps based on for example physical size or electrical charge.

However, because of time and stability constraints, certain compromises had to be made and the step with arguably the best resolution, the isoelectric focusing step (4.4), was in fact the most damaging of the steps with large losses occurring that made further purification impractical. As a direct consequence of this the major thrust of this study was directed to stabilizing the enzymes and verifying data that had been reported for less stable preparations of the enzymes, and it was not possible to address the

question of the homology of the FPP and β-CPA synthases.

The investigation into optimizing the mycelial yields of the enzymes and the stability of the enzymes themselves examined several aspects. These included the rupture of the mycelium (3.3.2), the elements of the reconstitution and purification buffers (3.4) and the use of ion exchange as a purification procedure (4.3) as well as maximizing the specific yield (units/ g mycelium) of the enzymes in the fungus (3.3.4).

Several different nitrogen sources for the culture medium were investigated and a concentration of 10 g/l of sodium aspartate ascertained to be the most suitable nitrogen source for the preparation of stocks of mycelium for the purification of the enzymes (3.3.3). The results of the investigation raised several interesting questions that were however unfortunately ancillary to the main object of this project. Why does aspartate-based medium give a higher specific yield of enzyme when the relative nitrogen concentration was only 71 % of that of the 9 g/l nitrate-based medium (1.05 g/l as opposed to 1.49 g/l respectively) ? What is the effect of using sodium as the counter ion in place of potassium or say calcium ? Cress et al (1981) found that calcium had a significant effect on the operation of the secondary metabolic enzyme, dimethylallyltransferase. However, having achieved a significant increase in the specific yield of enzyme from the mycelium, it was inopportune to examine these questions and attention was turned elsewhere.

Growths using mycological peptone as a nitrogen source (3.3.3) yielded no trace of α-CPA or β-CPA on the tlc plate ((c) & (d), figure 10), despite it showing a comparable absorbance of the chloroform extract to the α-CPA producing nitrate-based chloroform extracts ((a), figure 10). Unfortunately in this experiment no attempt was made to assay for the presence of β-CPA synthase. A subsequent attempt to reproduce this result yielded trace amounts of α-CPA and β-CPA. The assay in the second experiment was positive for β-CPA synthase with normal amounts of enzyme being found. However the growths for the latter experiment, where traces of α-CPA and β-CPA were found, were for 2 days only whereas the growths

that did not show any trace of the secondary metabolites were for a full five days. Two questions arise from these results : firstly what was the species in the mycological peptone chloroform extracts that absorbed so strongly at 280 nm and secondly if α-CPA and β-CPA were produced on day two then why was there no trace of these metabolites in either the filtrate or the mycelium on day five ?

The unstable and variable characteristics reported by McGrath et al (1976, 1977) for the enzymes and subsequently for the preparation of an acetone-dry ice powder by Herman (1985) led to an investigation into various methods of mycelial rupture (3.3.2). Homogenisation was disregarded after failing to successfully rupture the mycelium while an attempt to implicate the ice shear method of mycelial rupture in the instability of the enzymes was inconclusive. The latter method would bear repeating with similar pressures to those used by McGrath et al (1976, 1977). The method of rupturing the mycelium by preparing an acetone-dry ice powder was verified and the preparation of a freeze dried powder also successfully used. The latter method was found to give slightly more stable crude preparations with a 30 % increase in the specific activity of the enzymes over the former method, and so freeze drying was adopted as the method of mycelial rupture.

While EDTA was shown to destabilize the crude preparations (3.4.3) no conclusion can be inferred from the attempt to verify the insensitivity of β-CPA synthase to EDTA that was reported by McGrath et al (1977) (3.1.1). There are reports in the literature of similarly EDTA-insensitive secondary metabolic enzymes (see for example Heinstein, et al, 1971 and Cross et al, 1981 - p11 & 12 above). What was surprising about the results from this investigation was that the increase in β-CPA synthase activity obtained on the addition of 1 mM free EDTA to the assay mixture was only observed in the non-heat treated sample and was never observed in the heat treated samples. One would expect that 6 mM EDTA heat treated sample would show a similar increase to the non-heat treated sample. The result was however a complete inhibition of the β-CPA synthase in the sample with free EDTA

magnesium since magnesium was reported by McGrath et al (1977) to have little effect on β-CPA synthase while manganese had a detrimental effect.

The stability of the enzymes from the ion exchange column was a significant development (4.3) as was that of the increased ionic strength of the buffer. The previous reports of the purification of these enzymes have tended to use buffer strengths of 10 mM Tris HCl and may according to these results (3.4.6) have contributed to the instability of their preparations. McGrath et al (1977) began their purification with an ionic strength, $\mu = 0.02$ (≈ 20mM Tris HCl) but reduced it to $\mu = 0.01$ once the initial purification steps had been completed.

The molecular weight determinations (4.6) were only done twice. However the gel filtration column used for these determinations was the same one that had been used for the purification and the elution characteristics of the molecular weight determinations were typical of those obtained during the purification procedure in that the synthases eluted together with the isomerase trailing somewhat. Furthermore the variation reported by McGrath et al (1977) of the molecular weights from different mycelia was not found. Nor for that matter was an exceptional preparation obtained where the enzymes were completely stable, something that McGrath et al did obtain.

The molecular weights of the FPP and β-CPA synthases were very similar to each other and in complete disagreement with the 2:3 ratio (64 000 : 96 000) that McGrath et al (1977) found. Assuming that the fungal FPP synthase was dimeric since the vertebrate forms of the enzyme have been shown to be, then the 96 000 molecular weight reported by them for the β-CPA synthase would suggest a trimeric enzyme. If the β-CPA synthase were to have originated from a mutation of the FPP synthase, it is more likely that the dimeric structure of the FPP synthase would have been conserved and only the amino acid sequence of the subunits altered. Furthermore there have been almost no reports of the characterization of trimeric enzymes. The molecular weights of 83 000 and 77 600 obtained for the β-CPA and FPP synthases respectively are similar to those obtained for

vertebrate forms of FPP synthase (p11 above). Thus the β-CPA synthase could well be dimeric rather than trimeric and under these circumstances would have a greater possibility of being an evolutionary derivative from the primary metabolic enzyme, FPP synthase.

The biggest stumbling block to the homogeneous purification of the FPP and β-CPA synthases was the instability of the enzymes. While no empirical data can be given, it appeared that the harshness of the method of mycelial rupture was only partly to blame for the instability of the enzymes and that there could be another factor which is necessary for the stabilization of the enzymes that was lost at the start or during the early stages of the purification. Another alternative is that the enzymes have a short half life. The rapid fall in enzyme units from [illegible] (figure 11, p66) is indicative of such a short half life or to the metabolic *flexibility of the fungus. Metabolic flexibility* is essential in situations where there is a need to rapidly switch on or switch off selected enzymes *rapidly on the advent* of different metabolic circumstances. On the other hand one would anticipate that an enzyme from a fungal organism that is subject to a far greater degree of environmental change than say a mammalian enzyme would be more resilient to the rigors of in vitro handling. However the seven day effective life of the enzyme preparations, together with the poor stability on storage at -80°C of the enzymes, made purification for structural studies extremely difficult.

There are strong grounds for supposing that β-CPA synthase and FPP synthase could have been derived from a common genetic precursor. The enzymes compete for a common substrate, the cosubstrates for each enzyme (DMAPP & cAATrp) can in certain conformations mimic each other and the enzymes have similar molecular weights. Furthermore the fungus is eukaryotic and could well have multiple genes for the FPP synthase enzyme, thereby allowing a non-lethal mutation of one or more to form the β-CPA synthase enzyme. However this hypothesis can only be verified by the purification to homogeneity of the enzymes in question and the subsequent characterization of their subunit structure, amino acid compositions and primary structures and thereby determining the degree of homology between them.

APPENDIX

APPENDIX : CALIBRATION CURVES USING LINEAR REGRESSION

The Bradford method of protein determination requires the derivation of a calibration curve using BSA as a standard. For these protein determinations three concentrations were used - 5 µg/ml, 10 µg/ml and 20 µg/ml. A further control of 20 µg/ml lysozyme was used. The Bradford method (Peterson, 1983) assumes a similar binding constant for coomassie blue dye to all proteins irrespective of their shapes or sizes. Between the 5 µg/ml and 20 µg/ml BSA concentrations a plot of the absorbance against the protein concentration, given this assumption and the Beer-Lambert law, is linear. In other words the calibration curve is of the form

$$y = mx + c. \qquad (1)$$

Rather than plotting the points on graph paper and then fitting the curve with the naked eye, use was made of linear regression in the form of the least squares method to construct a calibration curve. Basically this method derives the slope (m) and the intercept (c) for the best fitting linear curve through the points of the standards and restates formula for corrected values of y given x. The method works thus :

$$\text{the slope} \quad m = \frac{\Sigma xy}{\Sigma x^2}$$

$$\text{and the intercept} \quad c = \frac{(\Sigma x^2 \Sigma y - \Sigma x \Sigma xy)}{N\Sigma x^2}$$

where N is the number of BSA standards, Σx is the sum of the concentrations of the BSA standards and Σy is the sum of their absorbances. Substituting m and c into equation (1) gives the equation

$$y_c = x\left(\frac{\Sigma xy}{\Sigma x^2}\right) + \frac{(\Sigma x^2 \Sigma y - \Sigma x \Sigma xy)}{N\Sigma x^2} \qquad (2)$$

Equation (2) can be used to derive the corrected absorbances of the BSA standards and with the absorbances of the unknowns their concentrations can be calculated.

Equation (2) was set up on a computer spreadsheet uring Lotus 123 to facilitate the derivation of the calibration curve. A sample printout is shown in figure 25. When a calibration curve was required, the spreadsheet was loaded and the absorbances of the standards and the control entered. The absorbances of the unknowns, together with their dilution factors, were also entered and the corresponding protein concentrations calculated by the spreadsheet.

Such an automated system is open to abuse that would result in the fitting of a straight line to points that by no means resemble a straight line. In this respect plotting the graph on graph paper would obviously reveal such a lack of correlation where as the least squares method would not. Thus to ensure that the calibration curve was straight and could be relied on, the standard deviation and the correlation coefficient were calculated (y is the actual absorbance measured and y_c the corrected absorbance derived from equation (2)). From (y_c - y) the standard deviation was calculated. The standard deviation as a percent of the total of the absorbances was also calculated. If this percentage was greater than 1 % or the correlation coefficient was more than 1.2 or less than 0.8, then the protein determination was regarded as not being linear and was repeated.

FIGURE 25 : A sample printout from the least squares spreadsheet for determining the protein calibration curve. The BSA concentration is (x) and the corresponding absorbance (y). For y_c, which is the corrected absorbance on which the calibration curve is based, only two significant places are shown on the printout but the calculation is to 3 significant places. The µl added column represents the volume of protein containing sample that was diluted to 1 ml with distilled water as required by the method.

LEAST SQUARES METHOD FOR CALIBRATION CURVES

Tube	BSA [C]	Abs	xy	x^2	y_c	y_c-y	$(y_c-y)^2$	y^2
1		104	0.52	25	0.10	.000	1.9E-34	0.0108
2		0.193	1.93	100	0.19	0.000	0.0E+00	0.0372
3	20	0.371	7.42	400	0.37	.000	3.1E-33	0.1376
Total	35	0.668	9.87	525		4E-17	3.3E-33	0.1857

Slope = 0.0178 Std dev = 2.3E-33

Intercept = 0.015 Std dev % = .00%

Corr. coefficient = 0.95

Lysozyme Abs = 0.34 [C] = 19.38 µg/ml

Tube No	*Absorbance*	Protein [C] / assay	µl added	Protein [C]
1	0.100	4.78 µg/ml	100	0.048 mg/ml
2	0.150	7.58 µg/ml	100	0.076 mg/ml
3	0.200	10.39 µg/ml	100	0.104 mg/ml
4	0.250	13.20 µg/ml	100	0.132 mg/ml
5	0.300	16.01 µg/ml	100	0.160 mg/ml

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