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Fatty alcohol oxidases involved in alkane-degradation by yeasts

by

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Finally, God, the heavenly father must be praised for making everything possible for me.

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LIST OF ABBREVIATIONS

3-D	three-dimensional
ABTS	2,2' azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AOX	alcohol oxidase
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1 propane
DEAE	diethylaminoethyl
FADH	fatty alcohol dehydrogenase
FALDH	fatty aldehyde dehydrogenase
FAOD	fatty alcohol oxidase
GMC	Glucose- Methanol- Choline
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2'-ethanesulphonic acid)
KDa	kilodaltons
MOX	methanol oxidase
M_r / MW	Relative molecular mass/ molecular weight
pI	isoelectric point
PMSF	phenylmethyl sulfonyl fluoride
QAE	diethyl-2-hydroxypropylaminoethyl
SEC	Size-exclusion chromatography
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl) aminomethane
mFAD	modified flavine adenine dinucleotide
YE	yeast extract

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CHAPTER ONE

A LITERATURE REVIEW

1.1 INTRODUCTION

Chemical and petroleum industries discharge into the environment a wide variety of organic wastes, especially hydrocarbons. Many organisms which are capable of utilizing a large variety of carbon sources available from places contaminated with such wastes have been identified and characterized. These organisms include bacteria, filamentous fungi and yeasts.

The degradation of aliphatic hydrocarbons by yeast and bacteria was studied mostly during the 1960's to 1970's (Wegner, 1990). During this period there was considerable interest in using the ability of bacteria to grow on C_1 compounds to produce single-cell-protein (SCP) from methanol. Studies were continued to determine if yeasts could also be grown on methanol. It was, however, found that utilization of methanol as the sole source of carbon and energy was restricted only to a limited number of species of the genera *Hansenula, Pichia, Torulopsis and Candida* (Wegner, 1990; Koutz *et al.*, 1989). The enzyme, methanol oxidase (MOX), is the first key enzyme for methanol metabolism in methylotrophic (methanol-using) yeasts. The enzyme is also capable of oxidizing lower primary alcohols and their halogenated derivatives, hence the name alcohol oxidase (AOX)(EC.1.1.3.13).

The ability of a wide variety of yeasts and moulds to utilize aliphatic hydrocarbons as growth substrates has also been established (Watkinson and Morgan, 1990), and the

pathway of oxidation is well documented. The utilization of n-alkanols to the corresponding fatty aldehydes by various alkane-degrading yeasts involves a long chain (fatty) alcohol oxidase (FAOD). This enzyme is different in its substrate specificity from the alcohol (methanol) oxidase of methylotrophic yeasts. A similar enzyme has, however, been reported in the cotyledons of jojoba seedlings (Kemp *et al.*, 1988).

Unlike the cytochrome P-450 and the peroxisomal β-oxidation systems, which have been studied extensively, very little is known about the nature of fatty alcohol and fatty aldehyde oxidising enzymes (FAOD and FADH, respectively). Originally it was assumed that both reactions were catalysed by NAD-dependent dehydrogenases. Later, however, the presence of FAOD and FADH were reported in *Candida guilliermondii* and *Torulopsis candida* (as cited by Blasig *et al.*, 1988). FAOD utilizes molecular oxygen and does not require an externally added cofactor.

The following brief discussion is just an overview of the data available in literature on the biochemical properties of MOX and FAOD from methylotrophic and alkane-degrading yeasts, including a comparison of the two enzymes with each other and with other oxidases wherever possible. The two enzymes will be referred to in this review as alcohol oxidase (AOX) with special reference to each, whenever such specification is needed.

1.2 THE MECHANISM OF INTRACELLULAR TRANSPORT OF HYDROCARBONS

The ability of microorganisms to grow on alkanes and other hydrocarbons has been attributed by many investigators to the presence of a lipophilic material on the walls of hydrocarbon-utilizing organisms (Bos and de Boer, 1967). In essence, it is presumed that the lipophilic nature of cell walls of hydrocarbon-grown cells facilitates direct contact between the hydrocarbon droplets and the surface layer of the cell walls. For example, in the yeast *Candida tropicalis*, the lipophilic material has been identified as a mannan fatty-acid complex, which aids in the capture and absorption of hydrocarbon droplets (Mallee and Blanch, 1977).

The mechanism of intracellular transport of hydrocarbons to their centres of oxidation is not yet well clarified. Presumptions have been made based on microscopical observations of microorganisms growing on hydrocarbon-containing media (Bos and de Boer, 1967; Mallee and Blanch, 1977). These observations have shown that yeast and bacteria accumulate unmodified hydrocarbon droplets in hydrophobic membrane enclosures found in their cell walls. A small hydrocarbon droplet passes through this area, which extends through the cell wall to the cell membrane, and then diffuses through the intracellular membrane system until it reaches a lipid inclusion or microbody.

It is then in the microbodies where alkane assimilation will take place since this organelle possesses enzymes, including FAOD, needed for conversion of alkanes to fatty acids. The fatty acids can then enter other known biological pathways to be further oxidized.

1.3 BIOCHEMICAL PROPERTIES OF ALCOHOL OXIDASES

1.3.1. Occurrence and substrate specificity

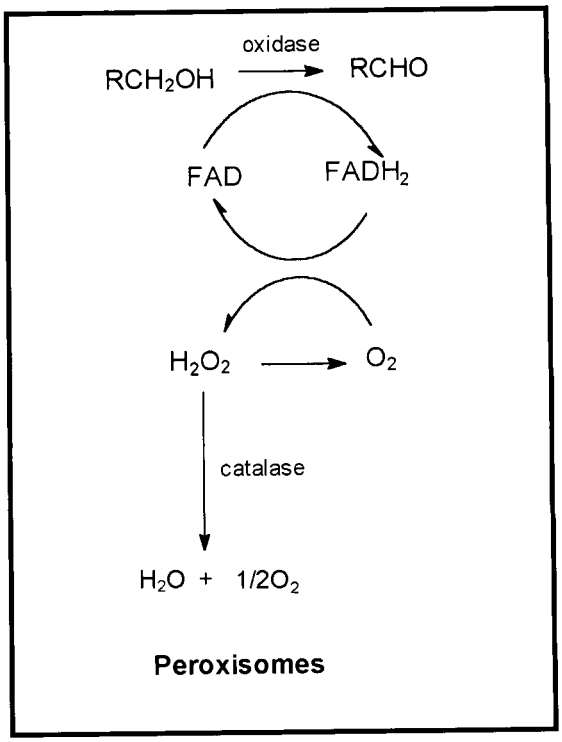
As already mentioned, alcohol oxidases are membrane-bound enzymes found in peroxisomes. Membrane fractions with alcohol oxidase activities for methanol and low molecular weight alcohols have been prepared from many methylotrophic yeasts, basidiomycetes fungi, as well as some bacteria (Daneel *et al.*, 1994) grown on these substrates.

FAOD activity is not only found in yeast cells grown on n-alkanes but also in cells grown on fatty alcohols and fatty aldehydes. Microsomal membranes having FAOD activity have been prepared from several yeasts such as *Candida tropicalis, Yarrowia lipolytica, Candida maltosa* and *Candida boidinii* (Hommel *et al.*, 1990, 1994; Il'chenko *et al.*, 1994; Dickinson *et al.*, 1992; Kemp *et al.*, 1988, 1990, 1991, 1994). Although the substrate specificity of FAOD varies among different yeasts, most of these enzymes will not oxidize carbon chains containing less than six carbon atoms. For example, FAOD from *C. tropicalis* was found to oxidize with decreasing effectiveness long chain diols, ω-hydroxy fatty acids, unsaturated fatty alcohols and branched chain unsaturated fatty alcohols in addition to long straight-chain alcohols (Kemp *et al.*, 1991). Similarly, the FAOD of *C. maltosa* has a broad substrate specificity, ranging from 1-alkanols of C_4 to C_{22} as well as α,ω-alkanediols, ω-hydroxypalmitic acid, phenyl alkanols, and terpene alcohols (Mauersberger *et al.*, 1992). In contrast, the FAOD specificity of *C. (Torulopsis)*

bombicola was found to be restricted to alcohols with carbon chains longer than that of heptanol (Il'chenko *et al* ., 1994).

1.3.2.The oxidation reaction by alcohol oxidase

Although many methylotrophic yeasts have been isolated during the last decade, it is only in *Hansenula polymorpha* and *Candida boidinii* where the methanol metabolism has been studied extensively (Ledeboer *et al.*, 1985). These studies have shown that the first step in metabolism of C_1 compounds involves the MOX catalysed oxidation of methanol to



When **R = H** the oxidase is **MOX**
When **R = C_nH_{2n+1}** the oxidase is **FAOD**

Figure1.1: Diagramatic representation of the oxidation of alcohols by alcohol oxidases inside yeast peroxisomes. When R = H the oxidase is MOX and when R = C_nH_{2n+1} the oxidase is FAOD (Adapted from Ellis *et al.*, (1985) and Mauersberger *et al.*, (1992)).

formaldehyde and H_2O_2. Formaldehyde is dissimilated further to CO_2 by formaldehyde dehydrogenase and formate dehydrogenase, generating H_2O_2 in the process. This H_2O_2 is broken down to H_2O and O_2 by catalase. The alternative route (which will not be described here) involves other enzymes and results in the assimilation of the carbon into cellular material.

The pathway of oxidation of long chain alkanes involves a sequence of oxidations at one of the two terminal C atoms, thereby producing the fatty alcohol ($R\text{-}CH_2OH$), aldehyde (R-CHO) and, finally, the fatty acid (R-COOH). ω-Hydroxylation and oxidation to the dioic acid is also common in the yeasts. The oxidation of the alcohols to aldehydes is accompanied by a stochiometric consumption of one mole of O_2, and a formation of one mole of H_2O_2. Figure 1.1 generally outlines the oxidation reaction of alcohols catalysed by MOX and FAOD.

1.3.3. The quartenary structure

The primary sequences of alcohol oxidases from many methylotrophic yeasts have been determined and it has been established that the enzyme is homologous to the flavoproteins glucose oxidase and cholesterol oxidase (Menon *et al.*, 1995). In fact, methanol oxidase and these two enzymes all belong to a family of proteins known as **g**lucose-**m**ethanol-**c**holine (GMC) oxidoreductases (Cavener, 1992). The overall structure of these enzymes can be divided into five distinct domains, namely the FAD-binding domain, an extended FAD-binding domain, a flavin attachment loop and an intermediate region, the FAD-covering lid, and a substrate-binding domain (Kiess *et al.*, 1998). The

general topology of the GMC oxidoreductases is conserved, with the exception of the presence of major inserts in two members of the group. There is an active site lid in cholesterol oxidase and some additional structural elements in the substrate-binding domain of alcohol oxidase. The two inserts in alcohol oxidase are 38 and 22 residues long, and since they are not in contact with the active site they are believed to play an important role in octamerization of the enzyme.

The quartenary structure of yeast alcohol oxidases ranges from dimers, to tetramers and even octamers. The following examples will illustrate this point very well. The methanol oxidases of *C. boidinii*, *H. polymorpha* and *Kloeckera sp.* are each a single octamer, whereas the alcohol oxidase of *P. pastoris* is made up of two octamers (Kato *et al.*, 1976; van der Klei *et al.*, 1991; Il'chenko *et al.*, 1994). Gel-filtration analyses of the FAOD of *Y. lipolytica* and *C. tropicalis* show that these two are each composed of two identical subunits, that is, they are dimers (Il,chenko *et al.*,1994; Dickinson and Wadforth, 1992). On the other hand, the methanol oxidase of *T. candida* is a tetramer (Il'chenko *et al.*, 1994). In each case however, the monomer contains one non-covalently bound prosthetic group, which is often flavin adenine dinucleotide (FAD).

In alcohol oxidases of many methylotrophic yeasts the FAD is present in a modified form (mFAD) which is a stereochemical modification of the natural enzyme and occurs spontaneously with FAD bound to alcohol oxidase. The presence of mFAD has a pronounced effect on the catalytic properties of alcohol oxidase in that the mFAD-containing enzyme has a significantly higher affinity for the substrate, and retains most of

its activity (Bystrykh *et al.*, 1998). Variable amounts of arabino-FAD are also present in alcohol oxidase and some of the flavin is found in the form of a semiquinone (Menon *et al.*, 1995; Kellog *et al.*, 1992). The enzyme alcohol oxidase is thus a flavo-protein.

The above properties are also observed among alcohol oxidases of many fungi such as *Peniophora gigantea*, *Polyporus obtusus*, and *Phanerochaete chrysosporium*, which show a strong relationship between these organisms and yeast (Daneel *et al.*, 1994).

Despite many attempts to obtain the three-dimensional structure of MOX, the current experimental approaches have not been able to produce very conclusive results so far (Vonck and van Bruggen, 1990,1992; Boteva *et al.*, 1999). The failure is probably due to the difficulty in obtaining the pure enzyme, the size of the enzyme, and ease of aggregation of precursor molecules in the cytosol. All efforts to localize the topogenic signal have also failed. No attempts have been made to elucidate the structure of FAOD. Elucidation of the 3-D structure would be valuable for further structural analysis such as determining the position of the active site(s) and the region involved in assembly. However, there are promising experimental approaches to consider in future such as site-directed mutagenesis to determine structure-function relationship, even though expressing genetically altered alcohol oxidase may affect targeting and/or import.

1.3.4. The effect of temperature and pH on enzyme activity and stability

The effect of temperature on alcohol oxidases isolated from different microbial species has been studied by subjecting the enzyme to various temperatures and measuring the enzyme activity and stability thereafter. The temperature optimum of MOX for methylotrophic yeasts is variable, ranging from 35°C for *Kloeckera sp.* to 45°C for *H. polymorpha* (Kato *et al*, 1976; Il'chenko *et al.*, 1994). For FAOD's the temperature optimum ranges from 25°C for *C. tropicalis* (Dickinson and Wadforth, 1992) to 38.5°C for *T. candida* (Il'chenko *et al.*, 1994). Activity gradually decreases at temperatures below the optimum, and is completely lost at several degrees above the optimum. Similar results are observed for the pH optima. Several physical properties are listed in a comparative fashion for a selection of oxidases in tables 1.1 and 1.2.

Table 1.1 Properties of methanol oxidases and fatty alcohol oxidases of some yeasts (Adapted from I'lchenko *et al.*, (1994)).

	Methanol oxidases				Fatty alcohol oxidases	
Properties	*Kloeckera sp.* (22)	*H. Polymorpha* (22)	*P. Pastoris* (21)	*T. candida* (21)	*Y. lipolytica* (21)	*C. tropicalis* (15)
MW (KDa)	673	669	675	290	140	145
MW of Subunits (KDa)	83	72	78	75	70	70
Act.energy (Kcal/mol)	5.98	10.8	11.1	27.4	nd	nd
pH optimum	8.0 - 9.0	8.0 - 9.0	7.3	7.6	9.3	9.0
T optimum ^{0}C	35	45	37	38.5	37	25
pI	nd	6.2	6.3	nd	5.5	nd

*nd = not determined
numbers in brackets denote reference number on the reference list.

Table 1.2. Properties of alcohol oxidases and glycerol oxidase from other organisms.

	Bacterial alcohol oxidase	Fungal alcohol oxidases		Glycerol oxidase
Properties	*Pseudomonas sp.* (22)	*Penicillium sp.* (14)	*Pleurotus eryngii* (18)	*Aspergillus japonicus* (21)
MW (KDa)	85	65	72.6	40
MW of subunits (KDa)	85	65	72.6	nd
Act.energy (Kcal/mol)	Nd	nd	nd	nd
pH optimum	10.0	9.0 – 10.5	5.0	7.0
T optimum ^{0}C	40	38	55	nd
pI	5.7	nd	3.9	nd

*nd = not determined
numbers in brackets denote reference numbers on the reference list.

1.3.5. Stereospecificity

The property of stereospecificity has been observed among fatty alcohol oxidases isolated from several fungal species. For instance, the FAOD of *Aspergillus flavipes* oxidizes cis-(z-) unsaturated fatty alcohols preferentially over the trans-(G-) isomers (Savitha and Ratledge, 1991). The FAOD of *C. tropicalis* also oxidizes unsaturated fatty alcohols more rapidly than the corresponding saturated alcohols (Dickinson and Wadforth, 1992). The same effect was also observed for the alkane-induced FAOD of *C. maltosa* which, during oxidation of 2-alkanols, demonstrated high stereoselectivity for the R(-) enantiomers (Mauersberger *et al.*, 1992). The preparation of an enantiomerically enriched α- substituted primary alkanol using a methylotrophic yeast alcohol oxidase was reported

by Clark *et al.* (1994). In this reaction a racemic mixture of 2-methyl-1-butanol was oxidized using a soluble alcohol oxidase from *C. boidinii*, and the enzyme showed preference for the S-enantiomer. Enantioselective alcohol oxidation by MOX has also been reported from *C. boidinii* by the same author. This enzyme oxidises (R)- and (S)-{1-^{3}H}-ethanol with preference for the removal of the *pro*-1-R hydrogen.

1.3.6. Light sensitivity

Fatty alcohol oxidase activities of microsomal membranes isolated from several alkane grown yeasts have been observed to rapidly decrease upon exposure to light, with the rate of inactivation being dependent on the intensity and wavelength of incident light, as well as the duration of the exposure. Photo-inactivation studies carried out by Kemp *et al.* (1990) on the FAOD's of *C. tropicalis* and *Y. lipolytica* revealed that these two alcohol oxidases are inactivated by exposure to light, with the latter being more susceptible. Hommel *et al.* (1994) also discovered that FAOD of *C. (Torulopsis) apicola* was photo-labile with approximately 80% activity loss upon exposure to daylight for 30 min. The FAOD of *Aspergillus flavipes* is also light sensitive, showing 60% activity loss on exposure to sunlight for 10 min (Savitha and Ratledge, 1991). There are no literature reports of light sensitivity of MOX.

According to Kemp *et al.* (1990) the photo-inactivation of FAOD may be explained as follows. Firstly, the prosthetic group found in the enzyme may be sensitive to light when oxygen is present. Secondly, this prosthetic group produces oxygen radicals that destroy amino acid residues at the active centre; and thirdly, radicals that are produced by a group

that is not part of the enzyme may destroy residues at the active centre. The three mechanisms proposed above have not been verified, and thus remain speculative. The only thing known at present is that the photo-inactivation phenomenon does not occur *in vivo*. This suggests that the inactivation is chemical, since there is no apparent change in the growth of yeast on n-alkanes in light compared to growth in dark.

1.4.7. Effect of substrate concentration, solvent, metal ions and inhibitors.

Studies of the effects of increasing methanol substrate concentration on MOX have been carried out on *H. polymorpha* (Bystrykh *et al.*, 1991) and *C. boidinii* (Anderova *et al.*, 1993). It was found that in a methanol-limited chemostat culture, methanol concentrations greater than K_i (the inhibition constant) for growth sharply decreased MOX activity in the yeast cells. This was attributed to dissociation of FAD from the enzyme molecules resulting in a chemical modification of FAD and the protein molecule. On the other hand, after complete consumption of methanol from the growth medium there is an increase in alcohol oxidase activity. The enzyme contains mainly the modified FAD (mFAD), the amounts of which stochiometrically correlate with a decrease in unmodified FAD levels. In other words, the kinetic properties of alcohol oxidase are dependent on the presence of the different forms of FAD, whose concentrations in turn depend on substrate concentrations. This characteristic may represent an unusual mechanism of adaptation to substrate-limited growth conditions, and it is possible that this observation may also apply to MOX and FAOD of other yeasts.

The effects of metal ions and inhibitors on yeast alcohol oxidases have also not been studied in detail except for the following published observations. The FAOD of *C. tropicalis* (Kemp *et al.*, 1988) was not inhibited by pyrazole or decanethiol, both of which are known to inhibit FADH and horse liver dehydrogenases respectively. The FAOD of *C. maltosa* was also not inhibited by 3mM KCN (Mauersberger, *et al.*, 1992). The FAOD of *C. apicola* was completely inactivated by 2mM β-mercaptoethanol and 8mM KCN (Hommel *et al.*, 1994).

There are no reports in literature of the effect of metal salts on the activity of yeast alcohol oxidases, but the following has been reported for alcohol oxidases from other organisms. The methanol oxidase of the ligninolytic fungus, *Peniophora gigantea*, was completely inhibited by 1mM concentrations of $FeSO_4$, $NiCl_2$ and $CuSO_4$ (Daneel *et al.*, 1994). The polyvinyl alcohol oxidase of the bacteria *Pseudomonas vesicularis var.povalyticus PH* is strongly inhibited by 1mM concentrations of Fe^{2+}, Hg^{2+} and Sn^{2+} and weakly by 1mM concentrations of Cu^{2+}, EDTA and thiourea (Kawagoshi and Fujita, 1997).

Assays of FAOD activity in *C. apicola* have shown that complete saturation of the reaction buffer with N_2 resulted in a reversible inhibition of FAOD (Hommel *et al.*, 1994). The use of DMSO as a solvent in the same system for the water-soluble substrates did not affect the enzyme activity. In contrast, acetone caused diminished enzyme activity. Due to the decrease in the water-solubility of reactants and products as the chain length and number of branches increase, alcohol oxidase activity also decreases. This is

observed particularly where either the substrate or product precipitates thereby exposing the enzyme to the inhibitor. The solution to this problem is the use of a non-aqueous solvent in which both reactants and products are more soluble.

1.4 THE ALCOHOL OXIDASE GENE (MOX AND FAOD)

Flavin-dependent alcohol oxidases have been isolated from a number of different fungal sources. The substrate specificity of the enzymes differs considerably depending on the source of the enzyme. Like all other peroxisomal enzymes studied so far alcohol oxidase is encoded by a nuclear gene and the enzyme is synthesized on free polysomes in the cytosol. Several MOX genes have been isolated and sequenced. These include *AOX1* and *AOX2* from *P. pastoris* (Koutz *et al.*, 1994), *MOX* from *H. polymorpha* (Lederboer *et al.*, 1985), and *AOD1* from *C. boidinii S2* (Sakai *et* Tani, 1992). Long chain alcohol oxidase has been purified from *C. tropicalis* (Dickinson and Wadforth, 1992), however, only very recently three FAOD genes from two *Candida* yeasts have been sequenced by Vanhanen *et al.* (2000). They described the first known DNA sequence encoding FAOD from any source and claim that these sequences present a new family of genes present in both prokaryotes and eukaryotes.

Oxidation of fatty alcohols by alcohol oxidases has been reported in germinating seedlings of the jojoba plant, which accumulates long-chain fatty alcohol-containing waxes as its main storage product. The utilization of these long-chain alkanes as well as long-chain fatty acids requires an appropriate oxidative pathway, presumably having ω-oxidation as a key component. However, to date, no potential long chain alcohol oxidase

DNA coding sequences have been identified in plants. Much of our current knowledge about yeast species capable of utilizing alkanes and long chain fatty acids comes from the study of *Candida* yeasts. Similarly very little is known at molecular level about long chain alcohol oxidases and aldehyde dehydrogenases from these organisms and other alkane-degrading yeasts. In order to expand and compliment the limited knowledge available about these organisms Vanhanen *et al.* (2000) managed to isolate and sequence two classes of FAOD genes (*fao1* and *fao2*) from a *C. cloacae* yeast. The same group has isolated a *fao1*-like coding DNA sequence from a *C. tropicalis* cDNA library using the *C. cloacae fao1* as a probe. Vanhanen *et al.* (2000) named these genes *fao* because they believe they code for long chain fatty acid alcohol oxidase.

Alignment comparison of the three *Candida fao* genes showed large regions of homologous domains throughout the full length of the derived sequences. Further analysis of the *Candida fao* sequences showed the highest sequence homology to genes of unknown assignment/function in *Arabidopsis* and *Mycobacterium*. High homology in domain V to other known oxidoreductases, particularly cholesterol oxidase, choline oxidase and cellobiose oxidase was also observed. These oxidases, just like alcohol oxidase from *Candida* yeasts are flavoproteins, use molecular oxygen and generate H_2O_2 in their reactions. These characteristics further confirm that both FAOD and MOX of the methylotrophic yeasts belong to the **g**lucose-**m**ethanol-**c**holine (GMC) group of oxidoreductases and flavoproteins. Of much interest is the fact that even though so little is known about the FAOD enzymes, these enzymes do not appear to belong to a totally new (or undiscovered) group of enzymes. Examples of GMC oxidoreductases include the

H. polymorpha and *P. pastoris* MOX's, *Drosophila melanogaster* glucose dehydrogenase, *Aspergillus niger* glucose oxidase and *E. coli* choline dehydrogenase (Cavener, 1992). In fact this group of enzymes well demonstrates the flexibility of evolution to produce enzymes with diverse catalytic activities while retaining the same overall architecture. Perhaps the 3-D structure of the ancestral protein of this family was such that the protein could bind a number of substrates, but, with low catalysis for each. Then high specificity displayed by the extant enzymes could be a result of subtle mutational refinements.

CHAPTER TWO

INTRODUCTION TO THE PRESENT STUDY

Although the growing interest in alkane utilization by yeast has led to extensive studies being carried out on the biochemistry and physiology of alkane-utilizing yeasts, only a small number of enzymes involved in alkane-utilization have been identified and characterized. The P-450 monooxygenases of the alkane-degrading yeasts have been the subject of extensive studies whereas very little is known about the enzymes involved in the subsequent oxidation steps and about the control and organization of the genes coding for these enzymes. An understanding of the degradation of very long chain alkanes ($>C_{20}$) has received virtually no attention, probably due to the difficulties associated with working with such highly insoluble and hydrophobic substances. As a result until recently no fatty aldehyde dehydrogenase or fatty alcohol oxidase has been cloned or characterized.

A literature study on yeast alcohol oxidases has also revealed that the long chain (fatty) alcohol oxidase (FAOD) from alkane–grown *C. tropicalis*, *C. maltosa*, and other alkane–assimilating yeasts accepted a broad range of n-alkan-1-ols. This property distinguishes the FAOD enzyme from the alcohol (methanol) oxidases of the methylotrophic yeasts which are only capable of oxidizing primary alcohols ranging from C_1 to C_5 in chain length (as cited by Mauersberger *et al.*, 1992). Earlier work on FAOD has shown that this enzyme is membrane-bound, light sensitive and exhibits some stereoselectivity (Kemp *et al.*, 1990; Hommel and Ratledge, 1990; Dickinson and Wadforth, 1992; Mauersberger *et al.*, 1992). Very few attempts have been made to purify the enzyme FAOD (Dickinson

and Wadforth, 1992; Il'chenko *et al.*, 1994) and this may be attributed to the photo-labile nature of the enzyme, making it quite difficult to work with, especially for longer periods. As a result many questions which cannot be resolved by working with crude preparations of the enzyme still remain unanswered. These include the mechanism of action of this enzyme, and the prosthetic group involved in catalysis and light sensitivity. The property of stereoselectivity has not been given any significant attention. Since oxygen acts as the electron acceptor in the oxidation reaction catalyzed by FAOD, the possibility of using this enzyme commercially in the synthesis of long-chain aldehydes seems feasible. It is worth mentioning at this point in time that when our project started in 1998 the *fao* gene sequences obtained by Vanhanen *et al.* (2000) were not yet available. As such, all the work in our project was done based on the fact that no FAOD gene had been sequenced and it was not possible to design any experiments based on DNA sequence information.

Certainly, the results referred to above indicate that the purification of FAOD was necessary for extensive biochemical characterization of the enzyme, as well as elucidation of its molecular properties. The ultimate aim of this project was therefore to study the FAOD genes in alkane-degrading yeasts and to construct a yeast system that will allow accumulation of desired long chain alcohols or aldehydes. As a result the project was initially divided into four parts namely;

1. Purification and characterization of FAOD enzyme from a strain of the yeast *Candida tropicalis*,

2. Obtaining a partial amino acid sequence of the enzyme and using the sequence to design primers or a DNA probe that will make it possible to locate the gene(s) encoding FAOD enzyme(s),
3. Construction of a genomic library and screening for FAOD gene(s),
4. Manipulation (mutation, gene replacement) of the gene(s) to investigate which of the products in the n-alkane assimilation pathway accumulates.

This dissertation is a report of the first part of the project, which is the purification of a FAOD from a strain of *Candida tropicalis*. Initially, the method of purification of this enzyme by Dickinson and Wadforth, (1992) was followed, but the attempt was not successful and therefore an alternative method was devised. With the very recent publication by Vanhanen *et al.* (2000) of the gene sequences of the long chain fatty acid alcohol oxidases from *C. cloacae* and *C. tropicalis*, it will be possible to modify the bigger project to move more quickly to genetic manipulation of the alkane-degrading yeasts.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Analytical chemicals and resins

Chemicals obtained from commercial sources were of analytical reagent grade and were used without further purification. Unless otherwise stated all reagents were obtained from either Sigma or Fluka. Difco laboratories supplied the yeast nitrogen base. Phenylmethylsulfonyl fluoride (PMSF) and calibration proteins for SDS-PAGE were obtained from Roche Diagnostics. Ammonium sulphate and dimethylsulfoxide (DMSO) were supplied by Merck. The following chromatography media were obtained from: (1) TosoHaas; DEAE-Toyopearl 650M, Toyopearl Super Q 650S and Toyopearl HW 50F (2) Pharmacia; QAE-Sephadex A50 (3) Affinity Chromatography Limited: MIMETIC^{TM} A6XL dye adsorbent ligands; Blue^{TM} 1, Blue^{TM} 2, Yellow^{TM} 2 and Hydrophobic Interaction Chromatography (HIC) ligands; Butyl agarose 4XL and Hexyl agarose 4XL.

3.1.2 Organisms

Five yeast isolates were screened for FAOD production. The isolates were obtained from the Yeast Culture Collection of the University of the Orange Free State as well as the Centraalbureau for Schimmelcultures. The isolates were *Candida tropicalis* OC3, *Candida tropicalis* CBS94T, *Candida tropicalis* OC23, *Candida maltosa* CBS5611T and *Yarrowia lipolytica* CBS599.

3.2 Methods

3.2.1 Enzyme assays and protein determination

3.2.1.1 Fatty alcohol oxidase assay

Fatty alcohol oxidase activity was determined spectrophotometrically at 405nm in coupled assays with 2,2' azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and peroxidase (Kemp *et al.*, 1988; Dickinson and Wadforth, 1992). The spectrophotometric assay is based on the methanol oxidase assay of Haywood and Large (1981). The method involves two successive enzymatic reactions (Verduyn *et al.*, 1984);

(i) Substrate + O_2 $\xrightarrow{\text{oxidase}}$ Product + H_2O_2 followed by,

(ii) H_2O_2 + ABTS $\xrightarrow{\text{peroxidase}}$ H_2O + $ABTS_{OX}$

The assay mixture contained in 3ml; 20mM glycine-NaOH buffer (pH 9.0), 0.8mM ABTS, 65µg peroxidase, 50µM decanol (substrate) and 200µl enzyme extract. The reaction rate was calculated using an absorption coefficient of $18400M^{-1}cm^{-1}$ for 1mM radical of ABTS (Kemp *et al.*, 1988) with two moles of radical cation formed per mole of substrate oxidized. The reaction was started by adding in a 3ml plastic cuvette, the ice-cold enzyme extract to the assay mixture previously equilibrated to 30^0C. The liberation of $ABTS_{OX}$ was then measured spectrophotometrically over a period of 120 seconds in a Genesis spectrophotometer set at 405nm. Fatty alcohol oxidase activity in U/ml (µmol product released.min^{-1}.ml^{-1}.enzyme) was calculated as follows:

$$U.ml^{-1} = \frac{V}{2 \times v \times \varepsilon \times d \times \Delta A.min^{-1}}$$

$$= \frac{3ml}{2 \times 0.2ml \times 18400M^{-1}.cm^{-1} \times 1cm \times \Delta A.min^{-1}}$$

$$= 0.408 \times \Delta A.min^{-1} \ U/ml$$

where V = total reaction volume (ml)

v = enzyme volume (ml)

ε = extinction coefficient of ABTS at 405nm = $18400M^{-1}.cm^{-1}$

d = light path of cuvette (cm)

$\Delta A.min^{-1}$ = change in absorbance per minute at 405nm

3.2.1.2. Fatty alcohol dehydrogenase assay

Fatty alcohol dehydrogenase assay was done following the method described by Mitsuyoshi and Tanaka (1990) with slight modifications. The assay is based on the principle that reduction of NAD^+ is measured at 30^0C with a recording spectrophotometer at 340nm. The assay mixture contained in a quartz cuvette 1.1ml of 50mM Tris-HCl (pH 8.5), 0.1ml each of NAD^+ (50mM dissolved in deionized water) and NaN_3 (50mM dissolved in deionized water), and 30µl substrate (50mM dissolved in 1,4-dioxan). The reaction was initiated by addition of 0.1ml enzyme. The reading was corrected for the blank which was obtained with enzyme and all the other reagents mentioned except for the substrate. Fatty alcohol dehydrogenase activity in U/ml (µmol product released $min^{-1}.ml^{-1}$.enzyme) was calculated as follows:

$$U.ml^{-1} = \frac{V}{v \times \varepsilon \times d \times \Delta A.min^{-1}}$$

$$= \frac{1.5ml}{0.1ml \times 6220M^{-1}.cm^{-1} \times 1cm \times \Delta A.min^{-1}}$$

$$= 2.41 \times \Delta A.min^{-1} \text{ U/ml}$$

where V = total reaction volume (ml)

v = enzyme volume (ml)

ε = extinction coefficient of NAD^+ at 340nm = $6220M^{-1}.cm^{-1}$

d = light path of cuvette (cm)

$\Delta A.min^{-1}$ = change in absorbance per minute at 340nm

3.2.1.3 Protein Determination

Estimates of protein concentrations were obtained by measurement of absorbance at 280nm as well as by using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985). The BCA method of protein assay was done using the commercially available kits supplied by Pierce, Rockford, IL, USA. Both the standard and micro assay kits were used, following the manufacturer's instructions.

3.2.2 Electrophoresis

3.2.2.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% resolving and 4% stacking gels (Laemmli, 1970). It was used to

monitor the purification process, to assess homogeneity and to determine the relative molecular mass (M_r) of the fatty alcohol oxidase, by comparing its electrophoretic mobility with those of standard proteins of known molecular masses.

Protein samples which were too dilute for direct loading on SDS-PAGE were either freeze-dried or concentrated using acetone containing 1% hydrochloric acid (at -20^0C or below). A 1:1 volume ratio of acetone to protein was centrifuged, redissolved and centrifuged again before finally being washed with 50% acetone (at -20^0C or below).

SDS-PAGE was performed using the "Mighty Small" SE 200 miniature slab gel electrophoresis unit, from Hoefer Scientific Instruments. The protein bands were detected with Coomassie Brilliant Blue R-250 and/or silver staining. Staining with Coomassie Blue was carried out as described in the Hoefer instruction manual and silver staining was performed according to the method described by Switzer *et al.* (1979).

The protein standards used were α_2-macroglobulin (M_r 170 000), β-galactosidase (M_r 116 400), fructose-6-phosphate kinase (M_r 85 200), glutamate dehydrogenase (M_r 55 600), aldolase (M_r 39 200), triose-phosphate isomerase (M_r 26 600), trypsin inhibitor (M_r 20 100) and lysozyme (M_r 14 300).

3.2.3 Yeast cultures

3.2.3.1 Optimization of yeast growth conditions

The five yeast isolates were cultured in triplicate in 20ml YNB-C16 broth in 100ml flasks. YNB-C16 broth contained 6.7g/l YNB and 10g/l hexadecane. The cultures were incubated at 30°C on a shaker and the optical density of each culture was read at 640nm (A_{640nm}) at 6-hour intervals. At the end of the experiment cells were harvested and the mass of cells obtained was recorded. The same experiment was repeated with 0.5% (w/v) yeast extract, added to the growth media.

3.2.3.2 Screening for FAOD production

The five yeast isolates (20ml cultures) were grown inYNB-C16 broth supplemented with 0.5%(w/v) yeast extract. The cells were harvested after 48h and disrupted in a mixture containing 1 volume cells to 1 volume of glass beads (450 – 600μm) to ½ volume buffer The buffer was 50mM N-(2-hydroxyethyl)piperazine-N'-(2'-ethanesulphonic acid (HEPES), pH 8.0. The mixture was vortexed in bursts of 1min alternating with 1min cooling on ice. The vortexing was done 5 times, 10 times, 20 times, 30 times and 40 times. The extent of cell disruption was monitored microscopically. The crude extracts containing both broken and unbroken cells were tested for FAOD activity using the ABTS assay. Cell debris was removed from the extracts by centrifuging at 3000*g* for 10min and both cell debris and the supernatant were again assayed for FAOD activity.

3.2.3.3 Subcellular localization of FAOD.

The supernatant obtained after cell disruption was subjected to several centrifugation steps at different speeds; 9 000*g*, 12 000*g*, 25 000*g* and 30 000*g*, to determine the correct speed to remove mitochondria, peroxisomes and microbodies. The pellet and supernatant obtained at each stage were assayed for FAOD activity and comparisons of the different pellets as well as the supernatants were made. Having established the presence of FAOD in the relevant fraction, conditions were maximized to capture all of the protein from the supernatant. This was done by centrifuging at the appropriate speed for different lengths of time (15, 30 and 60min), and comparing activity of FAOD in both the pellet and the supernatant.

3.2.3.4 Optimization of FAOD production in *C. tropicalis* OC3

To determine the best time to harvest the cells for maximum FAOD activity 20 x 20ml cultures in 100ml flasks plus five controls were prepared for *C. tropicalis* OC3 and incubated on a shaker at 30^0C as before. The controls contained everything found in the other cultures except hexadecane. Optical density (A_{640nm}) of the cultures was measured every 6h using one 20ml culture. The cells from this culture were harvested, broken and the crude extract was assayed for FAOD activity. The same was done with the control cultures.

3.2.4 Optimization preparatory to purification

3.2.4.1 Photostability of the FAOD enzyme

A membrane fraction containing FAOD activity to which 1mM PMSF had been added was divided into three equal volumes (2ml) in Eppendorf tubes. One tube (control) was covered in aluminium foil, and kept on ice in the dark, in a room at 4^0C. Another tube was kept on ice in the same room but exposed to fluorescent light. These two samples were assayed for FAOD activity at intervals. The third sample, which was kept at -70^0C, was assayed only once a week for a period of three weeks.

3.2.4.2 Effect of PMSF on stability of FAOD

Loss of FAOD activity in crude preparations of microsomal membranes, due to the action of proteases, has been reported before in literature. In order to confirm these reports the effect of PMSF on FAOD activity in crude extracts was studied using six equal samples (5ml) of cell-free extract. PMSF (1mM) was added to four of the samples while the other two were kept as controls. The first set of samples was kept on ice, in a room at 4^0C. This sample was assayed for remaining FAOD activity over a period of 100hours, while the other set (2 samples with PMSF plus 1control), stored at -70^0C, was assayed for FAOD activity once a week for a period of six weeks. The experiment was done in triplicate and was repeated twice.

3.2.5 Purification of fatty alcohol oxidase (FAOD)

All purification steps and methods were carried out at 0-4^0C with extracts protected from light because of the light sensitivity of the enzyme (Kemp *et al.*, 1990).

One article (Dickinson *et al.*, 1992) describing purification of FAOD, particularly FAOD from *C. tropicalis* was found in literature. This protocol as described in section 3.2.5.1 below was followed for purification of FAOD at hand. Only one alteration was made to this protocol namely, the use of QAE-Sephadex instead of QAE-cellulose, for the ion exchange step. The QAE-cellulose column was not available in our laboratory and was therefore substituted with QAE-sephadex (supplied by Pharmacia) since both chromatography columns are anion exchangers.

The protocol adopted from Dickinson *et al.* (1992) was found to involve too many steps which resulted in too much exposure to light and unnecessary loss of the enzyme at each step. At the end, even the minute quantity of enzyme available was still not pure. As a result quite a number of different purification methods were performed and assessed for suitability of developing a purification protocol for this enzyme. The methods are described below.

3.2.5.1 Purification protocol adopted from Dickinson *et al.* (1992)

In summary this method involved five major steps. First, the microsomal membrane pellet was obtained by breaking the cells and centrifuging the cell-free extract. The pellet was solubilized to release the enzyme from the membranes using a combination of two detergents, 0.5% (w/v) CHAPS and 1% (w/v) sodium deoxycholate. An ammonium sulphate precipitation step followed where the majority of contaminating proteins were precipitated out of solution with 24% (w/v) $(NH_4)_2SO_4$ and discarded. A 5.5% (w/v)

$(NH_4)_2SO_4$ was added to the resulting supernatant. The pellet thus obtained was used for purification of the FAOD enzyme. The FAOD active precipitate was then re-dissolved and applied onto a QAE Sephadex column. The final step was adsorption onto calcium phosphate.

3.2.6 Development of a new purification protocol

3.2.6.1 Optimization of solubilization of microsomal proteins with CHAPS

The detergent 3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) was added to 10ml aliquots of cell-free, crude enzyme extract to give different final concentrations. PMSF (50 mM) was added immediately to a final concentration of 1mM and the samples were incubated for 30min on ice. Thereafter, the samples were centrifuged (9000*g*, 10min) and percentage activity remaining in both pellet and supernatant was determined for each sample.

3.2.6.2 Optimization of ammonium sulphate fractionation

A 20ml FAOD crude sample obtained after solubilization of microsomal membranes was assayed and then subjected to ammonium sulphate precipitation in the following manner. Ammonium sulphate was added in increments of 5%(w/v) and after each addition the sample was centrifuged (10 000rpm, 10min). The supernatant and the pellet (re-dissolved in buffer) were assayed for FAOD activity. The addition of $(NH_4)_2SO_4$ stopped when FAOD activity could no longer be detected in the supernatant. The activity in both pellet and supernatant was expressed as a percentage of initial total activity of the sample. This

experiment was done in triplicate and was repeated twice on two different days. The activity of both pellet and supernatant was plotted against the percentage of $(NH_4)_2SO_4$ added.

3.2.6.3 Chromatography columns tested

All samples applied to chromatographic columns had passed through the ammonium sulphate precipitation step first and had been centrifuged (10 000rpm, 10min). These steps removed most of the contaminating proteins and thus ensured that the columns were not overloaded and blocked by the proteins. Fractions collected from columns were always 5 ml. All chromatographic methods tried for purification of the FAOD enzyme are explained below.

3.2.6.4 Ion-exchange chromatography

3.2.6.4.1 QAE-Sephadex A50

A 15ml sample was applied onto a previously equilibrated QAE-sephadex column (6cm x 2.5cm). The column was washed with 10mM carbonate-bicarbonate buffer, pH 9.2, until the absorbance values at 280nm were less than 0.02 and constant. The bound enzyme was eluted from the column by applying a linear gradient of the washing buffer (10mM carbonate-bicarbonate buffer, pH 9.2) containing 0.5M NaCl. All the collected samples were assayed for FAOD activity. FAOD active fractions were pooled together, assayed and protein concentration determined. A sample of this fraction was also kept for assessment of this step on SDS-PAGE at a later stage.

3.2.6.4.2 DEAE-Toyopearl 650M

In the first trial an FAOD active sample obtained from fractions pooled from QAE-Sephadex A50 column were loaded onto the DEAE-Toyopearl 650M column (2.5cm x 10cm) which had previously been equilibrated. The column was washed and gradient applied in exactly the same way as was done with QAE-Sephadex A50 column. Absorbance at 280nm was monitored as before and the fractions were assayed for FAOD activity. FAOD active fractions were pooled, assayed, protein concentration determined and a sample kept for SDS-PAGE. Since the DEAE-Toyopearl 650M column seemed to be a better purification step than QAE-Sephadex A50 column, this column was often used as the second purification step after $(NH_4)_2SO_4$ precipitation.

3.2.6.5 Affinity chromatography: MIMETIC™ A6XL affinity adsorbent ligands

3.2.6.5.1 PIKSI™ kit

The PIKSI™ (Affinity Chromatography Limited) kit was used to screen for the most suitable of the 10 Mimetic A6XL adsorbent ligands for purification of FAOD. The ten mini columns (containing 10 different dye ligands) were washed and equlibrated using 50mM Hepes buffer, pH 8.0, following the manufacturer's instructions. Then 2ml FAOD active samples eluted from the DEAE-Toyopearl 650M column and dialysed against a 50mM Hepes buffer, pH 8.0, were applied onto each of the 10 dye ligands. The columns were appropriately washed and the enzyme eluted (with equilibrating buffer containing 1M NaCl). The FAOD activity recovered from each column was calculated and

expressed as a percentage of the original activity applied onto the column. After the screening, three dye ligands, namely, Mimetic Blue™ 1, Mimetic Blue™ 2 and Mimetic Yellow™ 2, were selected for further testing.

3.2.6.5.2 Mimetic Blue™ 2 A6XL

The FAOD active fraction pooled from the DEAE-Toyopearl 650M column was first dialysed against 50mM Hepes buffer, pH 8.0, and then applied to the Mimetic Blue™ 2 A6XL column (5cm x 2.5cm) which had previously been equilibrated with the same buffer. The column was washed with the same buffer until A_{280nm} values of fractions collected (5ml) were less than 0.02. A linear gradient of the same buffer containing 1M KCl was then applied. All fractions collected were assayed and those with FAOD activity were pooled. The pooled fraction was assayed, its protein content was determined and a sample was kept to do SDS-PAGE at a later stage.

3.2.6.5.3 Mimetic Blue™ 1 A6XL

The Mimetic Blue™ 1 A6XL column (7cm x 2.5cm) was washed and equlibrated in exactly the same way as the Mimetic Blue 2 A6XL column. Then the total FAOD active fraction pooled (±100ml) after the Mimetic Blue 2 A6XL column was dialysed and loaded onto this column. Five ml fractions were collected and absorbance (A_{280nm}) monitored as described previously. The column was washed and a linear gradient applied as described for the Mimetic Blue 2 A6XL column above. All fractions collected were assayed, and the FAOD active fractions were pooled and the protein concentration was determined. A sample was also kept for assessment of the step on SDS-PAGE.

3.2.6.5.4 Mimetic yellow™ 2 A6XL

The Mimetic yellow A6XL column (7cm x 2.5cm) was washed and equilibrated in exactly the same way as the two Mimetic Blue columns above. A dialysed FAOD active sample (15ml) obtained from the DEAE-Toyopearl 650M column was loaded onto this column. The column was washed with the equilibrating buffer (50mM Hepes buffer, pH 8.0) until A_{280nm} values were constant and less than 0.02. A linear gradient of the same buffer containing 1M KCl was then applied. Fractions (5ml) were collected, assayed and those with FAOD activity were pooled. The pooled fraction was also assayed and the protein content was determined. A sample was kept for assessment of the step on SDS-PAGE later.

3.2.6.6 Hydrophobic Interaction Chromatography (HIC) adsorbent ligands

3.2.6.6.1 PIKSI™-H kit

The PIKSI™-H (Affinity Chromatography Limited) kit was used for rapid screening of all 10 hydrophobic interaction adsorbents. Each mini column was washed and then equilibrated with 50mM Hepes buffer, pH 8.0 containing 1M $(NH_4)_2SO_4$. Then 2ml FAOD containing samples obtained from DEAE-Toyopearl 650M column were dialysed and applied onto the columns. The columns were washed and enzyme eluted, again following the manufacturer's instructions. The enzyme activity recovered was then expressed as a percentage of the total activity applied onto each column. Following this

screening test two of the 10 hydrophobic interaction matrices, namely Butyl agarose 4XL and Hexyl agarose 4XL, were selected for larger scale purification of FAOD.

3.2.6.6.2 Hexyl agarose 4XL

The Hexyl agarose 4XL column was washed and equilibrated with 10mM carbonate-bicarbonate buffer, pH 9.2, containing 1.5M $(NH_4)_2SO_4$. Ammonium sulphate was added to a final concentration of 1.5M to a 20ml sample obtained from $(NH_4)_2SO_4$ precipitation. The sample was applied onto this column and 5ml fractions were collected. Absorbance (A_{280nm}) was monitored. The enzyme was eluted by application of a linear gradient of decreasing salt concentration, and 5ml fractions collected were assayed. FAOD active fractions were pooled and assayed. The protein content of the pool was determined and a sample kept for SDS-PAGE at a later stage.

3.2.6.6.3 Butyl agarose 4XL

The Butyl agarose 4XL column was washed and equilibrated in exactly the same way as the Hexyl agarose 4XL column. Then $(NH_4)_2SO_4$ was added to a final concentration of 1.5M to the 50ml FAOD active sample pooled from the Hexyl agarose 4XL column. The ammonium sulphate concentration of this sample was adjusted to 1.5M and the sample was applied to the column. The column was washed and the enzyme eluted as described above. A very low yield was, however, obtained and it was decided to apply 40% (v/v) ethylene glycol to the column in an effort to desorb any remaining FAOD activity. This step was successful. The two sets of FAOD active fractions were pooled separately, assayed and the protein content of each determined.

3.2.6.7 Size Exclusion Chromatography

3.2.6.7.1 Isolated FAOD enzyme on Toyopearl HW 50F

The Toyopearl HW 50F column (100cm x 1.5cm) was washed and equilibrated with 10mM carbonate-bicarbonate buffer, pH 9.2. Then 15ml FAOD active samples obtained from Butyl and Hexyl agarose 4XL columns were thoroughly dialysed with water to remove ethylene glycol and then applied to the Toyopearl HW 50F column. The column was then washed with the same equilibrating buffer. Two millilitre fractions were collected, assayed and those with FAOD activity pooled. The pooled fraction was also assayed and its protein content was determined.

3.2.6.7.2 Denatured FAOD enzyme on Toyopearl HW 50F

The Toyopearl HW 50F column (100cm x 1.5cm) was washed and equilibrated with 10mM carbonate-bicarbonate buffer, pH 9.2, containing 6M urea. FAOD active pools obtained by elution with 40% (v/v) ethylene glycol from either the Butyl or Hexyl agarose 4XL columns were dialysed to get rid of the ethylene glycol. Urea was added to the dialysed FAOD-active sample to a final concentration of 6M. The sample was applied onto the Toyopearl HW 50F column. The column was washed with the same equilibrating buffer and fractions (2ml) were collected, assayed and absorbance monitored (A_{280nm}). All fractions constituting one protein peak (as determined from the A_{280nm} values) were pooled. A 5ml sample was taken from each pool and dialysed thoroughly to remove the urea. Then 2ml samples of the dialysed fractions were taken and combined in an attempt to re-associate the denatured complex and recover activity.

All the different pools (dialysed and undialysed) obtained from this experiment were also run on SDS-PAGE.

3.2.7 Physico-Chemical Characterization

We consider the determination of pH and temperature optima for FAOD by the method described below not to be a suitable method in this coupled reaction, where two enzymes, FAOD and peroxidase are mixed together in the assay mixture. It is possible that the two properties being investigated for FAOD could well be the properties of the peroxidase. The ideal method for determining the FAOD pH and temperature optima would therefore be following the release of the alcohol on gas chromatography (GC), where the amount of alcohol released at different pH and temperature values would be a measure of FAOD activity at that particular pH or temperature value. It was however not possible to do these reactions using GC in our laboratory, and bearing in mind the draw-backs the pH and temperature optima for FAOD were determined as described below.

3.2.7.1 Optimum pH

Optimum pH of FAOD was determined over the pH range 6.0 to 11.0. The pH range was constructed by adjusting the pH values of the buffers used as follows. From pH 5-8 a 50mM Hepes buffer was used, and from pH 8-11 a 20mM glycine-NaOH buffer was used. The assay procedure was carried out as described previously. The enzyme assays were done in triplicate, together with a control at each pH, and the experiment was repeated three times on three different days.

3.2.7.2 Optimum Temperature

The optimum temperature of FAOD was determined over the temperature range 20^0C to 70^0C. At each temperature the reaction was started by addition of the ice-cold enzyme to the assay mixture which had previously been equilibrated to the required temperature. Assays were done in triplicates together with a blank at each temperature and the experiment was repeated three times on three different days.

3.2.7.3 pH stability

The assay mixture without substrate was adjusted to the required pH value using a strong acid or base. The enzyme was then added to the assay mixture and incubated at 20^0C in the dark. Aliquots were withdrawn at different time intervals and the reaction was started by addition of the substrate incubated at the same temperature. Assays were done in triplicate and the experiment was repeated three times on three different days.

3.2.7.4 Thermostability

Thermostability of the purified FAOD was determined at the following temperatures: 20^0C, 30^0C, 40^0C, 50^0C and 60^0C. The enzyme was added to the assay mixture without substrate at pH8.0 and the mixture was incubated at the required temperature in the dark. Aliquots were withdrawn at different time intervals and the reaction was started by addition of the substrate pre-equilibrated at the same temperature. Assays were done in triplicate at 30^0C and the experiment was repeated three times on three different days at each temperature. The initial activity of the enzyme before incubation was taken as 100%.

3.2.7.5 Substrate specificity

The substrate preference of FAOD was determined using the commercially available long chain (fatty) alcohols and other organic compounds. The substrates tested were both short and long chain alcohols ranging from C_5 to C_{22}. All substrates ranging from C_5 to C_{12} were prepared as 10mM solutions dissolved in DMSO, those longer than C_{12} were dissolved in pristane because of their insolubility in DMSO. The activity was assayed in triplicate and the experiment was repeated three times on three different days.

The effect of DMSO and 1,4-dioxan on FAOD activity was determined using dodecanol and decanol as substrates. This was to determine if the two solvents have any negative effect on the enzyme activity so as to be able to choose between the two.

The substrate specificity experiment was repeated using a different set of substrates and these substrates were dissolved in 1,4-dioxan. Substrates that could not dissolve in 1,4-dioxan were dissolved by heating them up in a 50^0C waterbath. Since most of the substrates used in this experiment dissolved in 1,4-dioxan , unless otherwise mentioned, all following experiments were done with substrates dissolved in this solvent.

3.2.8 Studies of FAOD and FADH induction in *C. tropicalis* OC3 cells

Three 20ml cultures of *C. tropicalis* OC3 yeast cells were prepared in YNB media with yeast extract (0.5%w/v) containing 1% (w/v) each of the following carbon sources; lauric acid, palmitic acid, dodecane, hexadecane, stearic acid and oleic acid. As a control, triplicates of 20ml cultures in YNB media containing no carbon source were prepared.

The cells were incubated at 30^0C and harvested after 42hrs of growth by centrifugation at 3000*g* for 5min. The harvested cells were weighed, broken with glass beads on a vortex machine and then centrifuged (3000*g*, 5min). The cell-free crude extract thus obtained was solubilized in 10mM CHAPS and centrifuged again (9000*g*, 10min). The solubilized fraction was used to carry out all the following studies:

3.2.8.1 Determination of FAOD cellular levels of *C. tropicalis* OC3

Using the ABTS assay the solubilized fraction obtained from cells grown on the different carbon sources was assayed for FAOD activity. The protein content of each fraction was determined using the BCA assay method. FAOD activity of each solubilized fraction was expressed in enzyme units per milligram of protein. The results are shown in figure 4.24 and are expressed as percentages relative to the solubilized fraction FAOD activity of the carbon source giving the highest FAOD activity.

3.2.8.2 Determination of FADH cellular levels of *C. tropicalis* OC3

The solubilized fraction obtained from cells grown on hexadecane, oleic acid and the control were assayed for FADH activity as described above. The isolated FAOD was also assayed for FADH activity. The results of this experiment are shown in figure 4.25.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Yeast Cultures and FAOD Production

4.1.1 Optimization of yeast growth conditions

Cultures of the five yeast isolates on a minimal medium containing only water, yeast nitrogen base (YNB) and 1%(w/v) hexadecane, resulted in very poor growth of the yeast cells (≤0.1g per 20ml culture). This was also reflected by the very low optical density values (A_{640nm}). However, addition of 0.5%(w/v) yeast extract (YE) to the growth medium resulted in fairly high A_{640nm} values and at least a three-fold increase in the yield of wet cells (≥ 0.35g per 20ml culture). The growth curves of *C. tropicalis* OC3 and *C. maltosa* CBS5611T isolates are shown in figure 4.1 below.

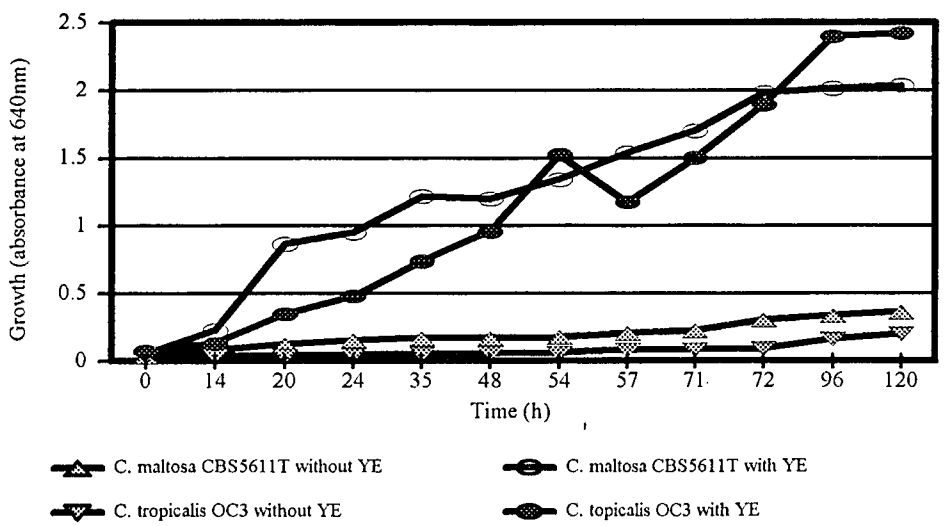


Figure 4.1 Comparison of growth of *Candida tropcalis* OC3 and *Candida maltosa* CBS5611T isolates in the presence and absence of yeast extract (YE).

Since it was established that addition of 0.5%(w/v) yeast extract to the growth medium improved growth of the yeast isolates, all yeast cultures prepared thereafter were supplemented with 0.5 %(w/v) yeast extract.

4.1.2 Screening for FAOD production

The 20ml cultures of the five yeast isolates were harvested and the cells were weighed and disrupted as described in section 3.2.3.2. Growth and FAOD activity were determined and the results were as shown in Table 4.1 below.

Table 4.1 Growth and FAOD activity of the 5 yeast isolates

Yeast isolates	**Wet cell mass(g) per 20ml culture**	**FAOD activity(U/ml)**
C. tropicalis OC 3	1.23	0.76
C. tropicalis OC23	0.83	0.00
C. tropicalis CBS94T	1.58	1.52
C. maltosa CBS5611T	1.31	0.71
Y. lipolytica CBS599T	0.78	0.00

Using the ABTS assay FAOD activity was only observed in three yeasts namely, *C. tropicalis* OC3, *C. tropicalis* CBS94T and *C. maltosa* CBS5611T. The other two yeasts, *C. tropicalis* OC23 and *Y. lipolytica* CBS599 did not show any FAOD activity. The latter yeasts were not used in further experiments. Contrary to what we observed above, *Y lipolytica*, has been reported to produce FAOD (Kemp *et al.*, 1990, 1994).) The failure to detect FAOD in *Y. lipolytica* and the other *C. tropicalis* yeast strain using the ABTS assay might be due to the fact that the assay was done at the wrong time (culture too old)

or because hexadecane does not induce FAOD activity in these strains. Fatty alcohols can also be oxidized by dehydrogenases (Ueda and Tanaka, 1990; Sahm *et al.*, 1982; Lebeault *et al.*, 1970). It has been shown previously (Kemp *et al.*, 1994) that the majority of FAOD's are thermolabile and photosensitive and in particular the FAOD of *Y. lipolytica* was more photolabile. Therefore accidental exposure of these FAOD's to light and heat during disruption of the cells could also have resulted in loss of FAOD activity in the two yeasts.

Note from examiners reports: One of the external examiners commented that the ABTS assay can be influenced by the presence of catalase, which also decomposes H_2O_2. Unfortunately we never added any catalase inhibitors to the ABTS assay. This might explain why no FAOD activity was observed with some yeast strains. It might also explain the relatively low FAOD activities observed in most of the experiments with *C. tropicalis* OC3.

4.1.3 Optimization of cell disruption

Conditions for optimal cell disruption with glass beads were determined. The minimum number of vortexing cycles (one minute vortexing followed by two minutes cooling on ice) required to give reasonable activity from about 0.35g of cells was found to be 20. Fewer vortexing cycles left too many cells unbroken. Thirty vortexing cycles gave the best results whereas 40 resulted in slightly less activity. Figure 4.2 shows how the number of vortexing cycles influenced the release of FAOD activity from the yeast cells. The buffer used for cell disruption was 50mM HEPES, pH 8.0.

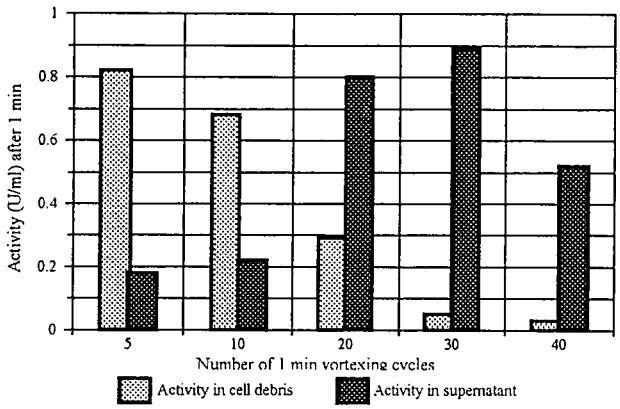


Figure 4.2. The effect of vortexing *C. tropicalis* OC3 cells with glass beads on the release of FAOD activity into the supernatant.

4.1.4 Subcellular localization of FAOD activity.

The cell-free crude extract obtained after cell disruption was subjected to several centrifugation speeds to determine the correct speed to separate the different cellular components and to find the sub-cellular fraction containing most of the FAOD activity. The highest FAOD activity was obtained in the microsomal fractions of *C. maltosa* and *C. tropicalis* OC3 (Figure 4.3). For *C. tropicalis* CBS94T even though the activity was

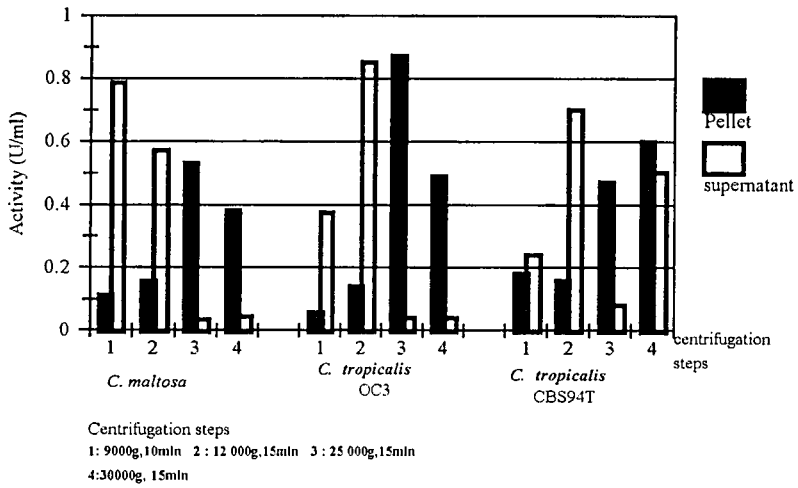


Figure 4.3 Localization of FAOD activity in membrane fractions by centrifugation at different speeds.

highest in the microsomal fraction, the activity remaining in the supernatant was relatively high even after centrifuging at the highest speed for the longest time. This may suggest the presence of a soluble FAOD in this yeast. A soluble alcohol oxidase was reported by Il'chenko *et al.* (1994) in *Y. lipolytica*. It is more normal to find a peroxisomal FAOD than a soluble one since these kinds of enzymes are mostly known to be membrane-bound. For this reason this yeast was also dropped from this study.

Having established the presence of FAOD activity in the microsomal fraction of the three yeast isolates, conditions were maximized to capture as much as possible of the FAOD activity in the pelleted microsomal fraction. Figure 4.4 shows that the highest FAOD activity was obtained in the pellet by centrifuging the supernatant containing this fraction for 60 min at 30 000*g*. It was, therefore, finally decided that the best method to obtain a microsomal fraction with high FAOD activity was by removing mitochondria by centrifugation at 9000*g* for 10 min and then pelleting the microsomal membrane fraction containing FAOD activity by centrifuging the remaining supernatant at 30 000*g* for 60min.

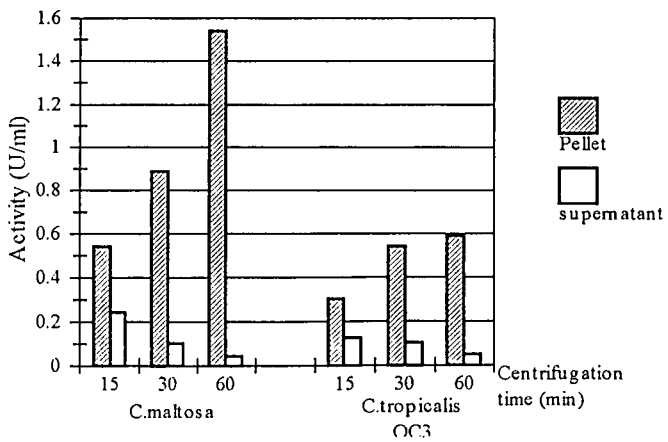


Figure 4.4 Determination of the length of centrifugation time at 30 000*g* required for capturing the FAOD active microsomal fraction maximally from the supernatant.

Later this method was however found not to work effectively when a large volume was centrifuged. A significant amount of FAOD activity still remained in solution and could not be recovered even after centrifuging for the longest time. As a result the supernatant obtained after removal of the mitochondria (9000g, 10min) was used for purification of the enzyme without prior pelleting of the microsomal fraction.

Note from examiners reports: It became clear from one of the examiners reports that the reason why no peroxisomal fraction but only a microsomal membrane fraction with FAOD activity was obtained, was that mechanical cell disintegration caused disintegration of the peroxisomes and partial solubilization of the FAOD.

4.1.5. Variation in FAOD activity during growth of *C. tropicalis* OC3

C. tropicalis OC3 was selected for further optimization and isolation of FAOD. Fig. 4.5 shows growth and FAOD activity for *C. tropicalis* OC3 grown on YNB-C16 supplemented with 0.5%(w/v) yeast extraxt. Absorbance values at 640nm, and hence the wet mass of cells, increased linearly from 0-72h after which it remained constant.

The production of FAOD also increased linearly until it reached a peak at 48h after which it gradually decreased. It should, however, be noted that subsequent experiments showed that the A_{640nm} values did not relate in any way to production of FAOD. For example, while in some instances the enzyme was produced from as early as 3h of growth the A_{640nm} values and wet cell mass were found to be very low at this time. Neither did high wet cell mass and high A_{640nm} values guarantee the presence of the enzyme in the culture.

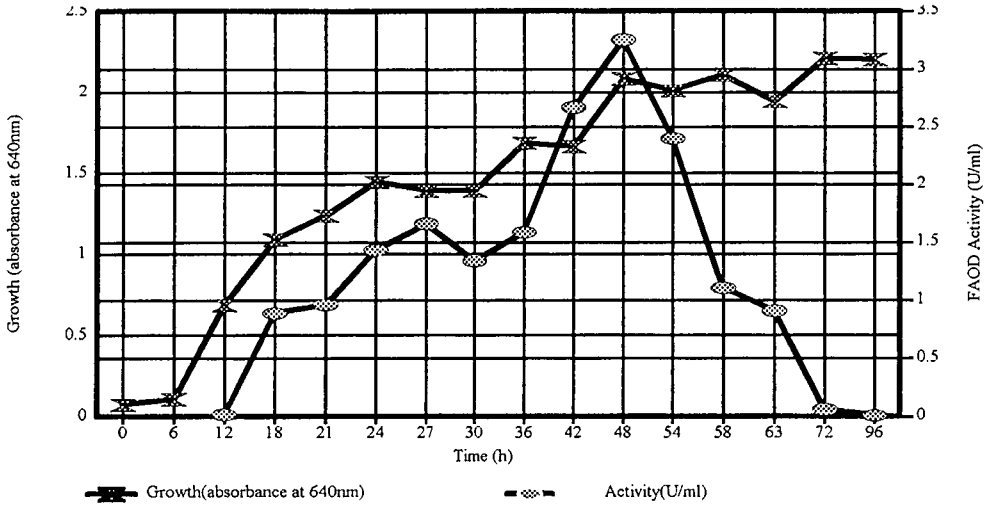


Figure 4.5. Growth and FAOD activity of *C. tropicalis* OC3 during the first 96h of growth on hexadecane in YNB medium supplemented with 0.5%(w/v) yeast extract.

In the example given in fig.4.5 it was established that optimum FAOD activity is reached after 48h growth on hexadecane in 100ml shake flasks containing 20ml YNB medium supplemented with 0.5%(w/v) yeast extract. Generally it could be said that regardless of the size of the culture the enzyme production never reached a peak before 12h of growth, and hence the cells were never harvested before 12h of incubation.

4.1.5 The effect of PMSF on the stability of FAOD

Loss of FAOD activity in crude preparations of microsomal membranes has been reported in literature and was also experienced in our early experiments. This loss of FAOD activity might be due to the action of proteases. To counteract the effect of these proteases the protease inhibitor PMSF was added to all membrane preparations. The presence of PMSF in preparations resulted in reduced deterioration of FAOD activity

thus enabling storage of samples for a longer period than was possible without the inhibitor (Fig.4.6). Samples kept at -70^{0}C retained at least 50% of their activity after 3 weeks.

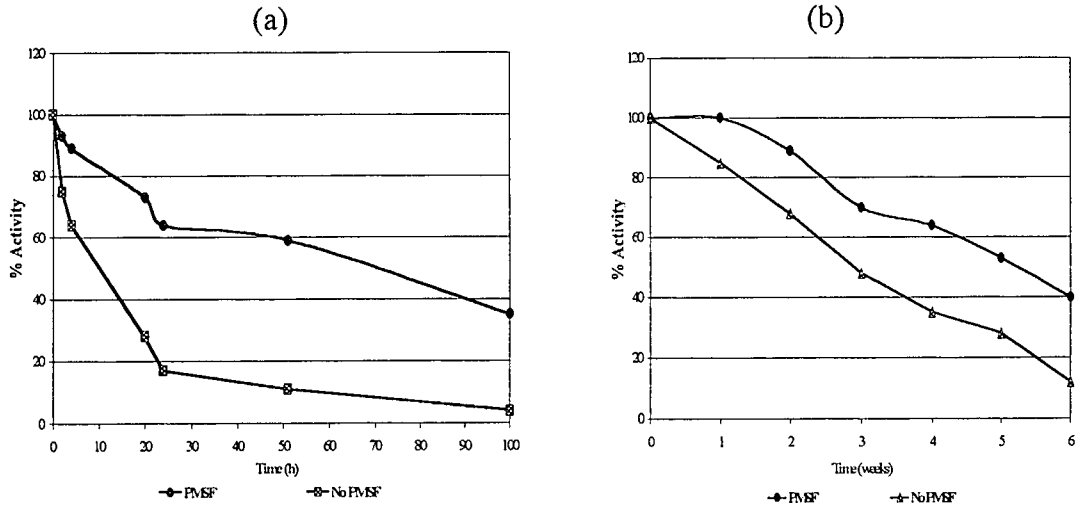


Figure 4.6 The effect of PMSF on FAOD stability, (a) samples stored on ice for several hours and (b) at –70^{0}C for several weeks.

4.1.5 Photostability of FAOD from *C. tropicalis* OC3

It has previously been reported that FAOD is light sensitive (Dickinson and Wadforth, 1992). Figure 4.7 shows that the membrane fraction that was exposed to light lost FAOD activity more rapidly than the one kept in the dark at the same temperature. Since light sensitivity studies had shown that crude cell preparations of *C. tropicalis* lose FAOD enzyme activity very rapidly when exposed to light, measures were taken to ensure that all samples were protected from light during purification of the enzyme. The membrane preparations being used for enzyme purification were also stored at –70^{0}C to further lengthen the life of the enzyme.

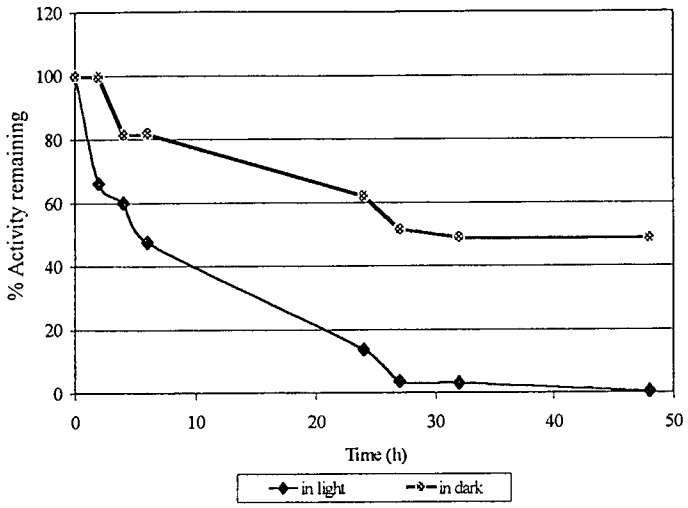


Figure 4.7 Photosensitivity studies done with crude membrane preparations of *Candida tropicalis* OC3 at 4^0C.

2. Purification of FAOD

4.2.1 Purification protocol adopted from Dickinson *et al.* (1992)

As already described the Dickinson et al. (1992) protocol involves the following major steps. First the microsomal membranes are solubilized with detergents, CHAPS and deoxycholate, followed by $(NH_4)_2SO_4$ fractionation. The sample obtained from the $(NH_4)_2SO_4$ fractionation step is then applied to the QAE-cellulose column. Finally the pure protein is obtained by calcium phosphate adsorption.

The first attempt at purifying FAOD was carried out using the method of Dickinson *et al.* (1992). First the microsomal membranes obtained from breaking the cells were solubilized to release the enzyme with the detergents 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS) (0.5%w/v) and sodium deoxycholate (1%w/v).

In our experiments solubilization of microsomal membranes using the two detergents separately and as a combination was effective and no problems were encountered.

The second step in the method was ammonium sulphate fractionation. The majority of contaminant proteins were precipitated out of solution with 24%(w/v) $(NH_4)_2SO_4$ and discarded followed by another precipitation with 5.5%(w/v)) $(NH_4)_2SO_4$ to capture the protein of interest. We found that the ammonium sulphate fractionation step had to be optimized because the published concentrations resulted in low yields of FAOD activity in our experiments.

Sodium deoxycholate had to be omitted from the purification buffer as it resulted in formation of a gel. As a result, even though CHAPS is considerably more expensive than cholate, the use of this detergent for solubilizing the microsomal membranes followed by $(NH_4)_2SO_4$ precipitation proved to be excellent as a purification step and was thus selected for use in purification of the FAOD enzyme.

Lastly, the enzyme was adsorbed from the FAOD active fractions obtained from the chromatography column using calcium phosphate.. Of all the purification steps described in this protocol the calcium phosphate adsorption step was the most difficult. The first problem one encountered with this step was the fact that the chemical formula of calcium phosphate, which appears only once in the whole article, is wrong (Ca_3PO_4). Therefore, one is faced with the tedious job of having to test all the different compounds with the name calcium phosphate (e.g $Ca_2(H_2PO_4)$, $Ca_3(PO_4)_2$...*etc*) in order to be able to find the

right compound. In screening for the right calcium phosphate it was found that FAOD activity of the test samples was rapidly lost upon contact with the compounds except for one, $Ca_3(PO_4)_2$. Adsorption of FAOD onto $Ca_3(PO_4)_2$ was found to be very poor with most of the enzyme still remaining in solution. Worse was the fact that even the minute quantities of FAOD that were adsorbed were not pure when assessed on SDS-PAGE. The calcium phosphate adsorption step was found to be just a waste of time and resources and therefore was immediately abandoned.

The FAOD elution profile on the QAE-Sephadex column (anion exchanger) is shown in figure 4.8. From this profile it can be seen that the entire protein sample loaded onto the column was not bound and no separation of FAOD from the contaminant proteins took place. This was of no use for purification of the enzyme and the step was also abandoned.

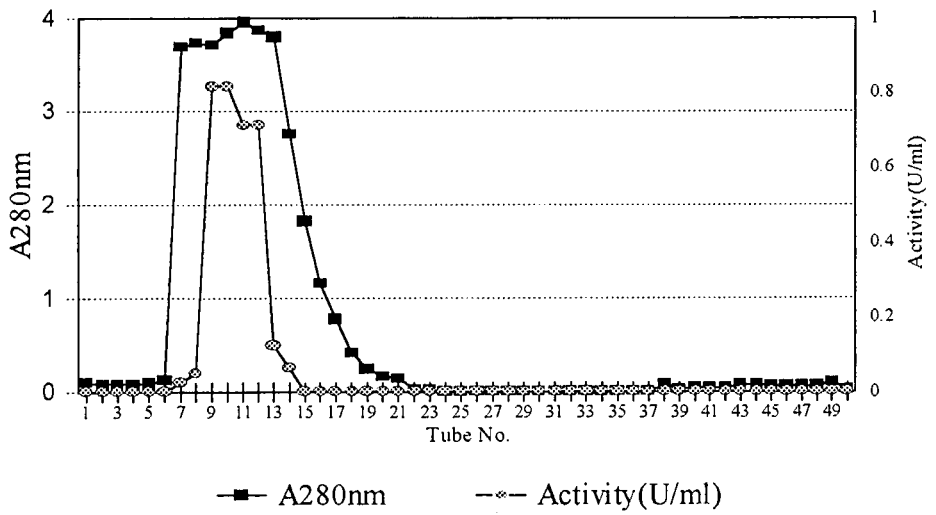


Figure 4.8 Elution profile of QAE-Sephadex chromatography of FAOD.

4.2.2 Development of a new purification protocol for FAOD

4.2.2.1 Solubilization of microsomal membranes

Having established from the Dickinson *et al.* (1992) protocol that the detergent, CHAPS, works well for solubilization of the microsomal membranes the next step was to determine the right concentration of the detergent to use. Therefore different concentrations of the detergent were added to 10ml samples of cell-free crude extract to which 1mM PMSF had already been added. The samples were incubated on ice for 30min and then centrifuged (9000*g*, 10min). The percentage activity in the residue and the supernatant was determined and compared for the different CHAPS concentrations. The experiment was repeated twice on two different days.

Figure 4.9 shows that the percentage activity in the supernatant increased with an increase in CHAPS concentration until it reached a point (10mM) where increasing concentrations of the detergent had no effect. Quite the opposite happened with the

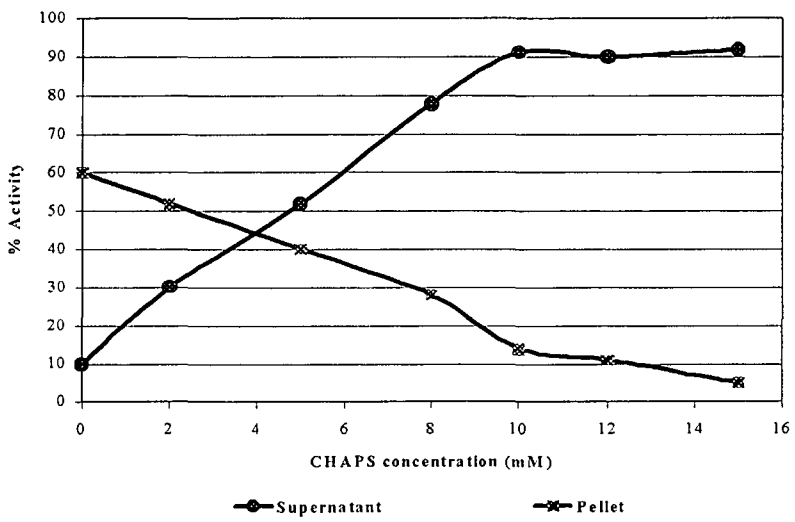


Figure 4.9 Determination of the concentration of CHAPS required for solubilization of microsomal membranes

residual activity. This therefore implied that a concentration of 10mM CHAPS was effective in solubilizing the microsomal membranes and releasing FAOD while any concentration higher than that had no effect on FAOD activity.

4.2.2.2 Ammonium sulphate fractionation of FAOD

The results of this experiment (fig. 4.10) show that FAOD precipitated in the range 10-30%(w/v) $(NH_4)_2SO_4$. Since at 40%(w/v) $(NH_4)_2SO_4$ all the FAOD was already precipitated, addition of more only resulted in precipitating more of the rest of the contaminanting proteins. As a result it was decided that for purification of FAOD 10%(w/v) $(NH_4)_2SO_4$ would initially be added to the supernatant after solubilization of the microsomal membranes. The resulting pellet would be discarded. This initial addition

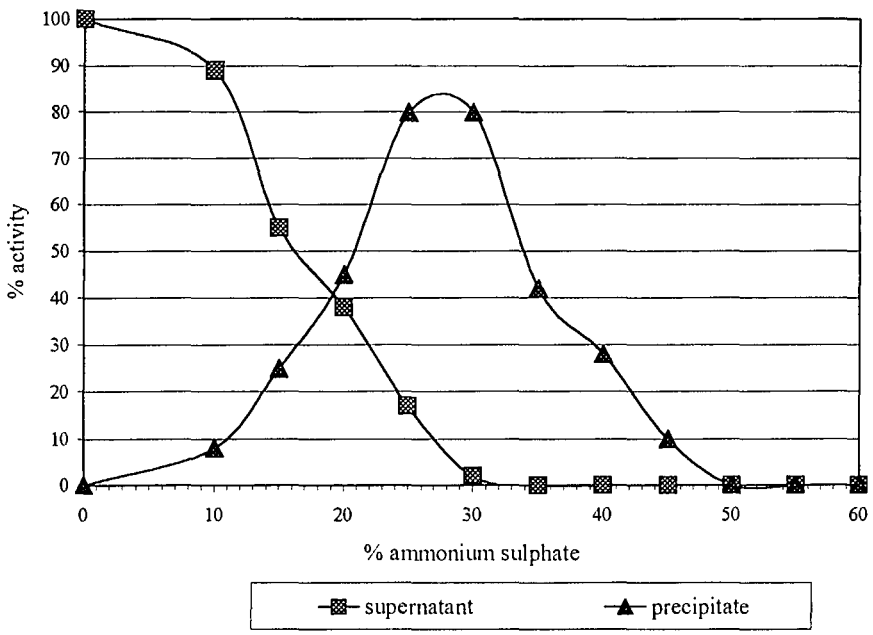


Figure 4.10 Ammonium sulphate fractionation of FAOD from cell-free crude extract.

of ammonium sulphate only served to remove a small amount of contaminating proteins and disintegrated microsomal membranes. FAOD was then precipitated from the supernatant by increasing the $(NH_4)_2SO_4$ concentration to 30%(w/v). Contrary to the same step in the previous protocol, this step managed to recover at least 80% of the total FAOD activity originally present in the crude extract. This therefore proves the effectiveness of the $(NH_4)_2SO_4$ precipitation step as a purification step in separating FAOD from a significant amount of the contaminating proteins.

Following optimization of the membrane solubilization with detergent and the ammonium sulphate fractionation steps a number of chromatographic columns were screened for suitability of purification of the FAOD enzyme. The following table (Table 4.2) is a list of 15 purification trials showing the different combinations of purification methods employed for purification of the FAOD enzyme.

Table 4.2 FAOD Purification Tables (Trials)

Purification attempt No.	Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	% Yield
1	Solubilization	135	24.2	2970	0.0081	1	100
	$(NH_4)_2SO_4$ precipitation	25	28.4	325	0.087	11	117.4
	DEAE-Toyopearl	90	18.4	72	0.26	32	76
	$Ca_3(PO_4)_2$ adsorption	4	0.27	2.6	0.11	14	0.011
2	Solubilization	100	84	3575	0.023	1	100
	$(NH_4)_2SO_4$ precipitation	20	87	385	0.23	10	103.6
	DEAE-Toyopearl	40	68	145	0.47	20	81
	$Ca_3(PO_4)_2$ adsorption	2	4.2	72	0.058	2.5	5
3	Solubilization	130	62	3900	0.016	1	100
	$(NH_4)_2SO_4$ precipitation	50	65	2000	0.033	2	95
	*Mimetic Blue™ 2	122(86)	96(35)	21(29)	4.6(1.2)	269(75)	54(56)
4	Solubilization	50	127	3200	0.04	1	100
	$(NH_4)_2SO_4$ precipitation	40	130	2000	0.065	1.6	102
	DEAE-Toyopearl	400	107	600	0.18	4.4	84
	Mimetic Blue™ 2	500	64	350	0.22	22	50
5	Solubilization	30	13	104.6	0.12	1	100
	$(NH_4)_2SO_4$ precipitation	10	15	31.4	0.48	4	115
	DEAE-Toyopearl	62	10.1	11.5	0.88	7.3	78
	Mimetic Blue™ 1	25	1.7	8.2	0.21	1.6	13

*Figures shown are for the minor trailing peak whereas figures in brackects are for the major activity peak.

Purification attempt No.	Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	% Yield
6	Solubilization	32	14.9	7500	0.002	1	100
	$(NH_4)_2SO_4$ precipitation	20	15.9	6400	0.0025	1.25	107
	DEAE-Toyopearl	10	3.5	400	0.0088	4.4	23
	Mimetic Blue™ 2	45	3.0	135	0.022	11	20
	Mimetic Blue™ 1	176	0.53	14	0.038	19	4
7	Solubilization	50	18.2	1045	0.017	1	100
	$(NH_4)_2SO_4$ precipitation	10	23	314	0.073	4	126
	DEAE-Toyopearl	120	10.1	11.5	0.88	52	55
	Mimetic Blue™ 1	38	1.7	8.2	0.21	12	9
8	Solubilization	35	23	1080	0.02	1	100
	$(NH_4)_2SO_4$ precipitation	10	24	450	0.053	2.7	104
	DEAE-Toyopearl	30	1.98	4.5	0.44	22	9
	Mimetic Yellow™ 2	12	0.38	1.2	0.32	16	2
9	Solubilization	120	23	960	0.02	1	100
	$(NH_4)_2SO_4$ precipitation	20	46.5	76	0.61	31	202
	Hexyl agarose 4XL	190	46.8	19	2.46	123	203
	Mimetic Blue™ 1	120	1.08	2.4	0.45	23	4.7
10	Solubilization	35	10.2	850	0.012	1	100
	$(NH_4)_2SO_4$ precipitation	17	14.5	50	0.29	24	142
	DEAE-Toyopearl	18	0.92	3.96	0.23	19	9
	Butyl agarose 4XL	16	0.05	0.42	0.12	10	0.5

Purification attempt No.	Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	% Yield
11	Solubilization	50	6.05	912	0.0066	1	100
	$(NH_4)_2SO_4$ precipitation	20	8.04	500	0.016	2.4	133
	Hexyl agarose 4XL	120	1.68	12.3	0.14	21	28
	Butyl agarose 4XL	38	0.152	0.48	0.32	48	2.5
12	Solubilization	120	2.5	960	0.0026	1	100
	$(NH_4)_2SO_4$ precipitation	35	0.74	236	0.0031	1.2	2.9
	Hexyl agarose 4XL(+40% ethylene glycol)	180	10.6	68	0.16	62	421
13	Solubilization	35	3.7	700	0.005	1	100
	$(NH_4)_2SO_4$ precipitation	15	1.3	360	0.004	0.8	35
	Hexyl agarose 4XL (+40% ethylene glycol)	100	1.8	140	0.013	2.5	49
14	Solubilization	20	6.0	400	0.015	1	100
	$(NH_4)_2SO_4$ precipitation	12	7.04	228	0.031	2	117
	Hexyl agarose 4XL (+40% ethylene glycol)	81	2.72	2.4	1.13	75	45
15	Solubilization	22	17.9	1140	0.016	1	100
	$(NH_4)_2SO_4$ precipitation	15	21.6	312	0.069	4	121
	Hexyl agarose 4XL (+40% ethylene glycol)	78	8.7	7.5	1.17	73	48.7

Looking at the above purification tables one realizes the fact that in the majority of trials the $(NH_4)_2SO_4$ precipitation step gave higher than 100% yield. This was probably a result of removal of some inhibitor by this purification step which then enhances the enzyme activity. Alternatively the precipitation step might have separated the catalase, which can interfere with the assay (see p42), from the FAOD. The concentration process also may have brought together all factors contributing to the stability of the enzyme, or the salt itself may somehow have stabilized the protein, which then results in enhanced enzyme activity.

Contrary to the precipitation step where a distinct pattern can be established, the purification factor and the specific activity values for the chromatographic steps are so variable no pattern can be made out of them. These values are a direct result of each step they represent and are supposed to show how effective the step has been in removing the contaminating proteins. In our case we believe while these values may be reflecting on the purification steps, they were however severely affected and reduced by the thermolability and light sensitive nature of this enzyme. Some loss of activity was also experienced during thawing of frozen samples. Loss of enzyme activity as purification progresses from one step to another is normal and was expected in this case. However, normal loss of activity combined with activity loss due to exposure to light had a dramatically negative impact in this case. Starting with a small volume meant less handling time and hence less time of light exposure resulting in higher purification and specific activity values. Starting with a large volume meant higher activity at the beginning, which also meant better yields of activity. However, dilution of the enzyme

sample from one step to the next meant an even larger volume which required longer time to run through the chromatographic columns and hence greater loss of activity from an overexposed dilute sample. While starting with a small volume seems to have been the answer to this light exposure problem it should also be noted that this was successful only if the starting sample itself already had a high enzyme activity. This was not always the case due to the variable peak of enzyme production in yeast culture. All in all one can say that while all precautions were taken to protect the enzyme against heat and light a considerable loss of activity during thawing of samples and due to exposure to light was unavoidable and thus resulted in the variable purification factor and specific activity values.

4.2.2.3 Ion -exchange chromatography (DEAE-Toyopearl 650M)

The elution profile (Fig. 4.11) shows that a lot of contaminating proteins were removed by DEAE-Toyopearl anion-exchange chromatography. The major activity peak was well separated from the bulk of the contaminating proteins with a 15% yield and a purification factor of 6 (Table 4.2). Assessment of this step on SDS-PAGE showed, however, that a lot of contaminating proteins were still associated with the FAOD protein. As a result the DEAE-Toyopearl 650M step was used subsequent to the $(NH_4)_2SO_4$ fractionation step in the subsequent purification trials while the search for more effective purification method(s) continued.

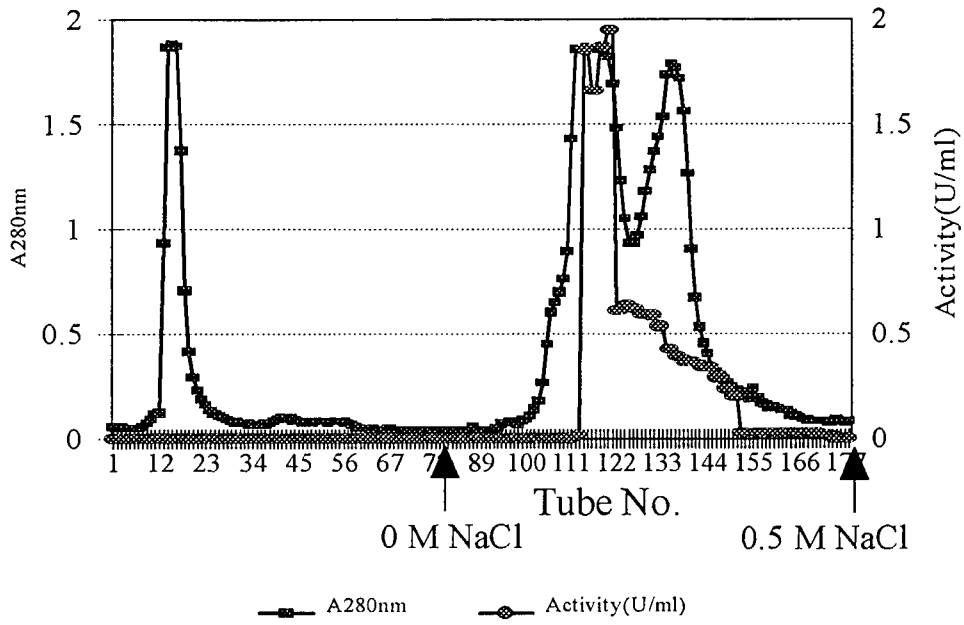


Figure 4.11 DEAE-Toyopearl 650M chromatography elution profile of FAOD. The arrows indicate the start and end of the NaCl gradient.

4.2.2.4 Affinity Chromatography - MIMETIC^{TM} A6XL dye ligands

Ten MIMETIC^{TM} dye ligands were screened for purification of FAOD using the Piksi^{TM} kit. The results are shown in Figure 4.12. Three Mimetic dye ligands, namely Mimetic^{TM} Blue1, Mimetic Blue 2 and Mimetic Yellow 2, were subsequently selected for purification of the enzyme. The Mimetic affinity columns were used following the DEAE-Toyopearl 650M step. The FAOD elution profiles on the dye columns are shown in figure 4.13 (a), (b) and (c) The Mimetic Blue 2 gave the most promising separation and even the SDS-PAGE showed a nearly pure enzyme. However, when one looks at the FAOD elution profile on this column (Fig.4.13 (c)) this is not such a good profile. A minor trailing tail of activity follows the major activity peak. It is this tail of activity which showed a pure enzyme on the SDS-PAGE gel (Fig.4.14 (a) lane B) whereas the

major activity peak where most of the FAOD enzyme was still showed several enzymes (Fig.4.14 (a) lane A). Even the figures shown in Table 4.2 (Attempt No. 3) display this remarkable difference in the nature of the FAOD enzyme recovered from these two peaks. As a result Mimetic Blue 2 column was at the time not considered to be ideal for purification of the FAOD enzyme, and as such more chromatographic columns were tested in the hope of finding a better chromatographic column. It is obvious from the purification tables that the Mimetic Blue 2 column was the best of all the columns tried. Probably with optimization of a few conditions a reasonably pure enzyme could have been obtained from this step. It was unfortunate that due to time limit this column was never tried again.

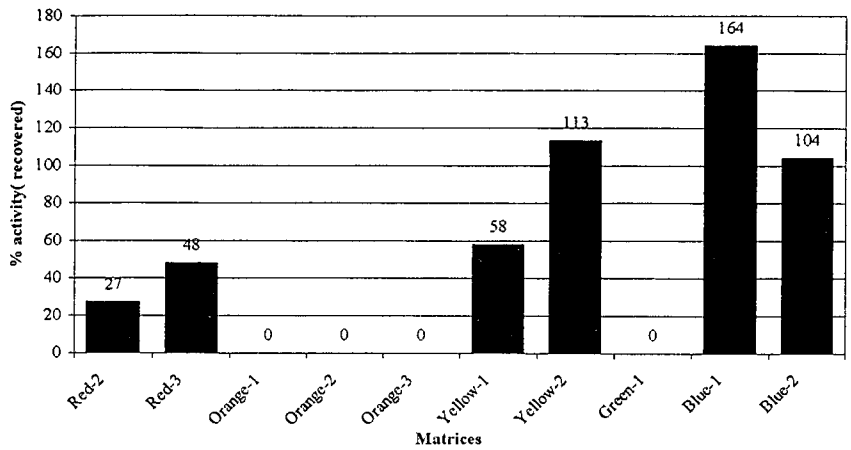


Figure 4.12 Screening of the Mimetic^{TM} dye adsorbent ligands for FAOD binding and release using the Piksi^{TM} kit.

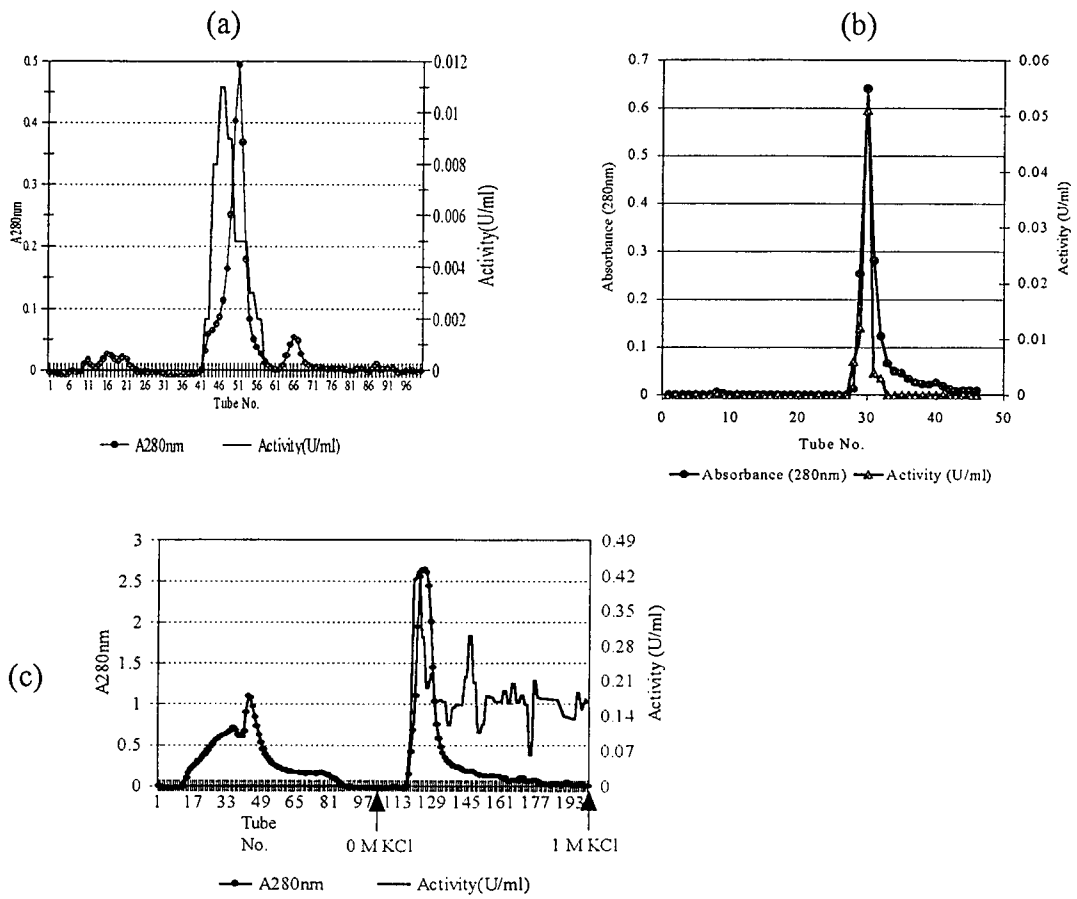


Figure 4.13 FAOD elution profiles on (a) Mimetic^{TM} Blue 1, (b) Mimetic Yellow 2. and (c) Mimetic Blue 2 affinity chromatography columns.

(a)

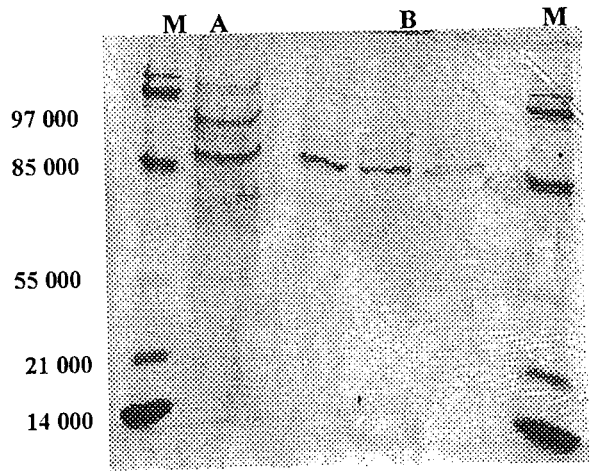


Figure 4.14(a) SDS-PAGE of FAOD active samples obtained from Mimetic Blue 2 column (Fig. 4.12(c)). **M** represents the marker lanes. **A** represents a sample from the major FAOD activity peak while **B** represents 3 different samples obtained from the tailing minor FAOD activity region.

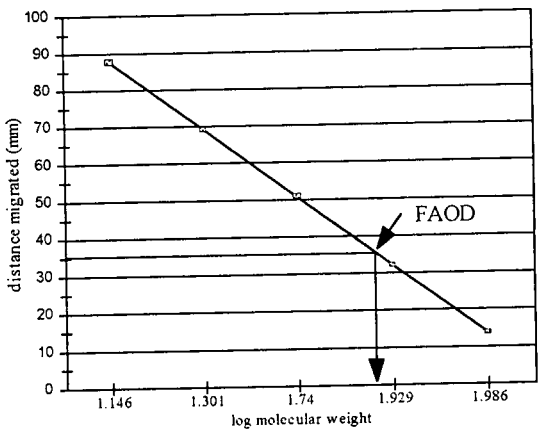


Figure 4.14(b) The protein molecular mass calibration curve.

According to figure 4.14(b) the relative molecular mass of the FAOD protein is about 85 000. Most FAOD's reported in literature have an M_r of 70 000 to 75 000, so our protein is of a larger size.

4.2.2.4.2 Hydrophobic interaction chromatography (HIC) using adsorbent ligands

The search for the best chromatographic column continued and hydrophobic interaction chromatography was employed. In order to choose the best HIC column the different columns were tested on a small scale using the Piksi^{TM}-H kit. Two HIC matrices, namely Butyl agarose 4XL and Hexyl agarose 4XL, were selected for purification of FAOD (Fig 4.15). Phenyl agarose 6XL was also good for purification of our enzyme, however, due to financial restrictions the resin was not available for use in our laboratory.

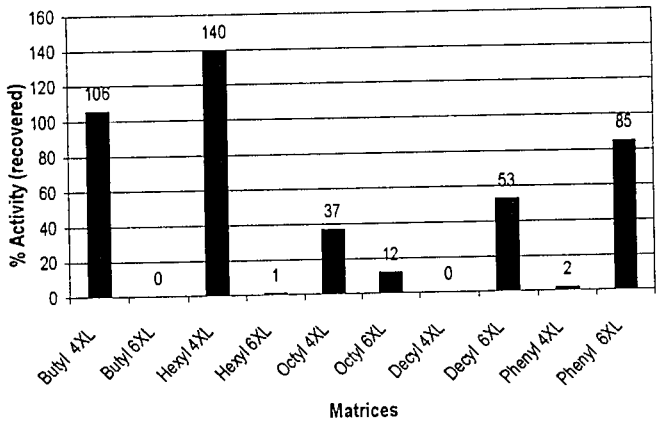
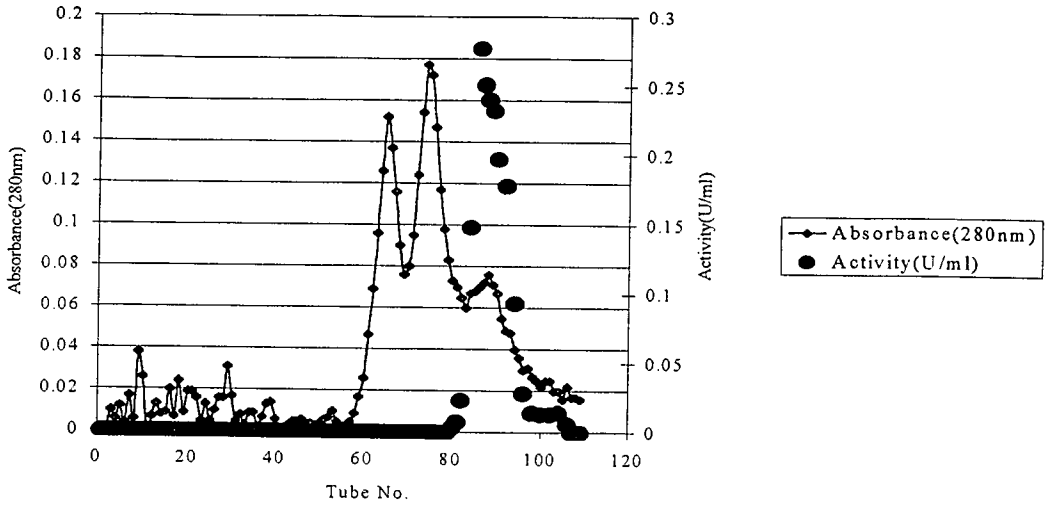


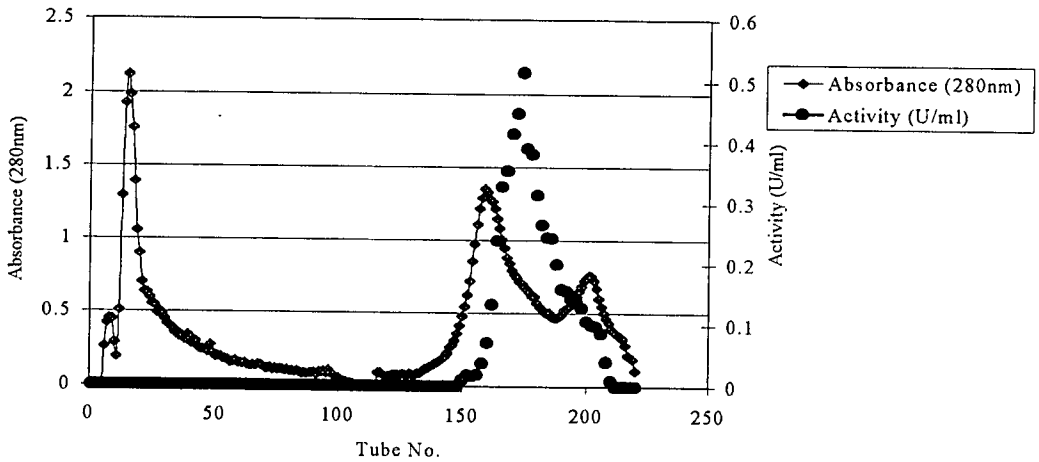
Figure 4.15 Screening of the Hydrophobic Interaction chromatography adsorbent ligands for FAOD binding and release using the Piksi^{TM}-H kit.

These columns were used in combination with other types of columns previously mentioned and the elution profiles are shown in figure 4.16 (a), (b) and (c). It is worth mentioning at this point that the difference in the performance of the different affinity columns is due to the difference in the extent of cross-linking in the immobilized alkyl/aryl group (e.g. 4XL vs 6XL) as well as the nature of the ligands (e.g. butyl vs phenyl).

(a)



(b)



(c)

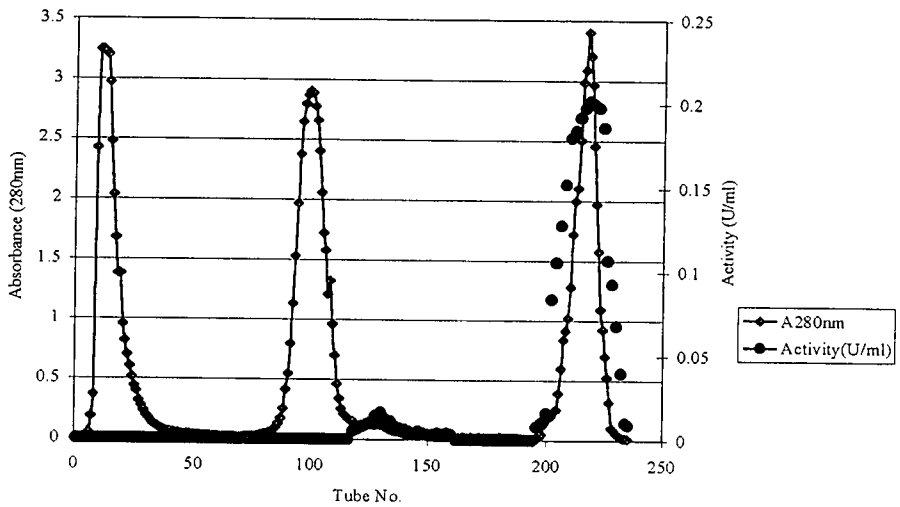


Figure 4.16 FAOD elution profiles on HIC columns (a) Butyl agarose 4XL, (b) Hexyl agarose 4XL and (c) Hexyl agarose 4XL eluted with 40%(v/v) ethylene glycol.

The last two purification attempts (Hexyl agarose 4XL columns)(table 4.2 attempts 14 and 15) were reproducible in terms of purification factor and specific activity values. In these two purification trials the specific activity value increased more than 70-fold and these values together with the elution profiles one would think that they suggest that the isolated FAOD enzyme is pure. However, judging of the homogeneity of an enzyme cannot be derived from elution profiles and purification factors as shown by the SDS-PAGE gels of samples obtained from these last two attempts (Fig. 4.17).

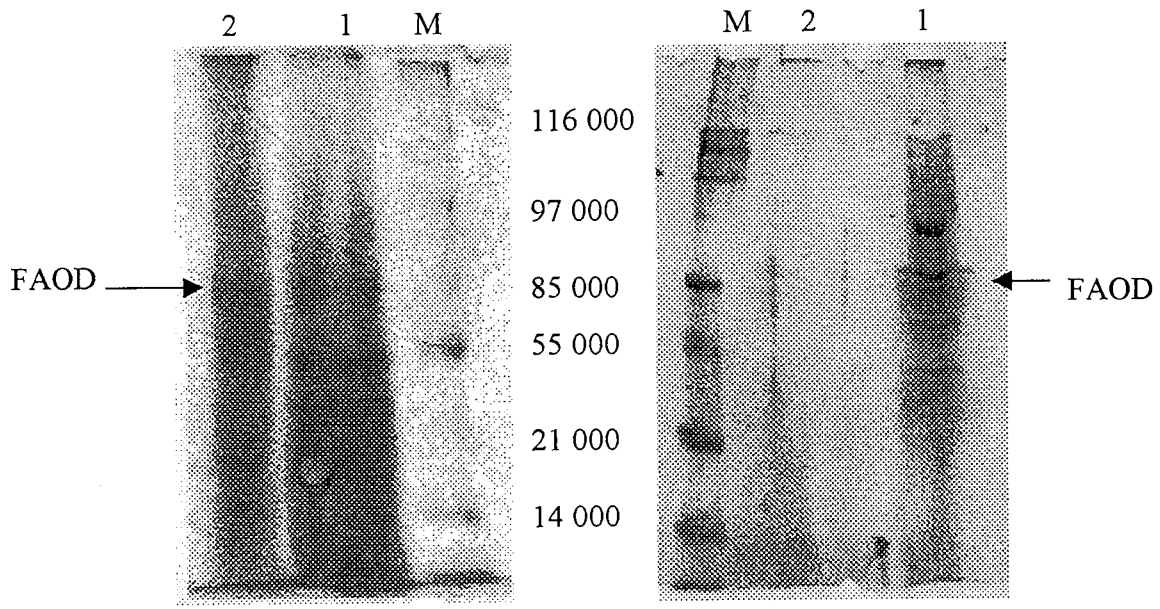


Figure 4.17 Some SDS-PAGE samples obtained from FAOD active samples eluted from the Hydrophobic Interaction Chromatography columns. **M** denotes the marker lane while lanes **1** and **2** denote the crude enzyme extract and the isolated FAOD enzyme respectively.

4.2.3 Size Exclusion Chromatography (SEC)

The FAOD protein sample obtained from the HIC column (Hexyl agarose 4XL) was applied to a SEC column. The elution profile is shown in figure 4.18(a). The profile shows only one protein peak corresponding to one FAOD activity peak. This implied that only a single protein is present or that the enzyme is an aggregation of protein subunits of the same size. When the FAOD sample was applied to the SEC column in the presence of a protein denaturant, 6M urea (Fig.4.18(b)), the protein peak eluted after the same number of fractions as before. No FAOD activity was however eluted. An exhaustive dialysis of the protein sample was done to remove the urea and hopefully reconstitute the protein complex. After the dialysis the sample still had no activity, which meant that the protein complex was irreversibly dissociated. This therefore implies that an intact protein

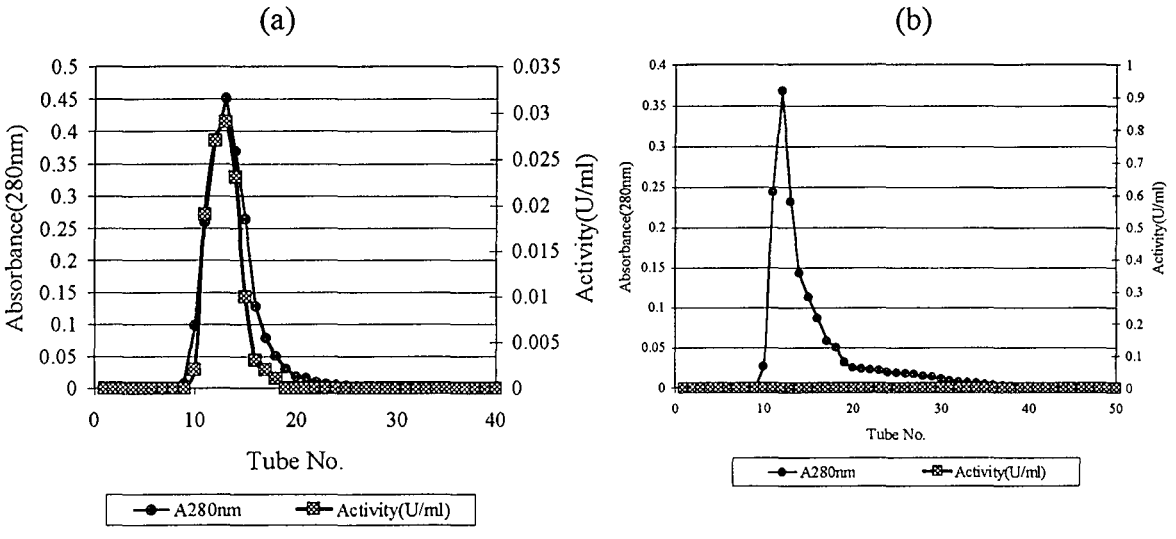


Figure 4.18 SEC of FAOD on Toyopearl HW 50F in (a) absence and (b) presence of 6M urea.

complex is necessary for activity. SDS-PAGE of samples from the SEC column in the absence of urea were no different from the Hexyl (or Butyl) agarose (Figure 4.18) from which they were obtained whereas a sample run in the presence of urea showed multiple

bands of variable molecular masses. The denatured protein has lost enzyme activity, but has the same size as the intact protein. This may imply that the denaturant (urea) only disturbs the active site of the enzyme or the binding site of the prosthetic group so that the disturbance results only in loss of activity whereas the size of the protein does not change. The dissociation of the flavin group from the protein would also not result in a significant alteration of the protein size if the flavin is a very small molecule.

4.2.4 Physico-Chemical Properties

Determination of the physico-chemical properties of FAOD was done using enzyme obtained from the Hexyl agarose 4XL column. Enzyme obtained from the major peak in the elution profile shown in Fig. 4.16(c) (tubes 200-230) was used in these experiments.

4.2.4.1 Optimum pH

The optimum pH of FAOD was determined as described in section 3.2.8.1. FAOD exhibits a broad activity range of pH 7.5 to pH 10.0 with maximum activity at pH 9.5 (Fig. 4.19). Membrane-bound (crude extract) enzyme showed similar characteristics.

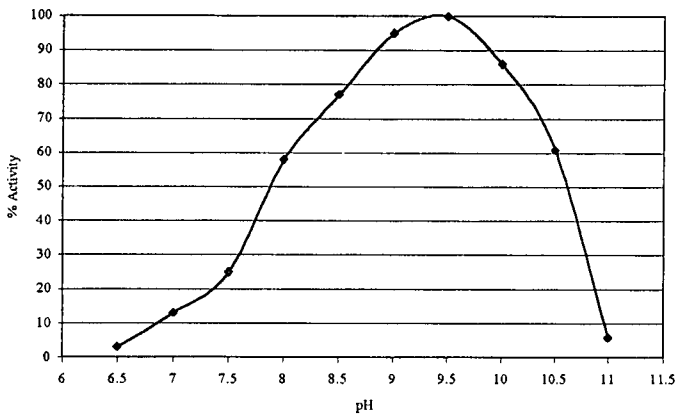


Figure 4.19. Determination of FAOD pH optimum.

The observed properties of the FAOD stated above are similar to the properties of FAOD purified by Dickinson *et al.* (1992). Since the pH optimum values are far different from those described by Kay *et al.* (1967) for peroxidase (pH 4.5-5.8), we believe that these values are true and pertain only to FAOD and not to peroxidase used in the assay. Thus the method employed for determination of FAOD pH optimum was not at fault.

4.2.4.2 Optimum temperature

FAOD activities were determined at different temperatures and the maximum activity was normalized to 100%. FAOD exhibited an optimum temperature of 35^{0}C (fig. 4.20). This optimum is slightly different from the 30^{0}C for FAOD from the same organism by Dickinson and Wadforth (1992). This value is also quite different from the stated temperature optimum for the peroxidase which is 20^{0}C at pH 6.0 (Welinder *et al.*, 1979, Maehly *et al.*, 1955).

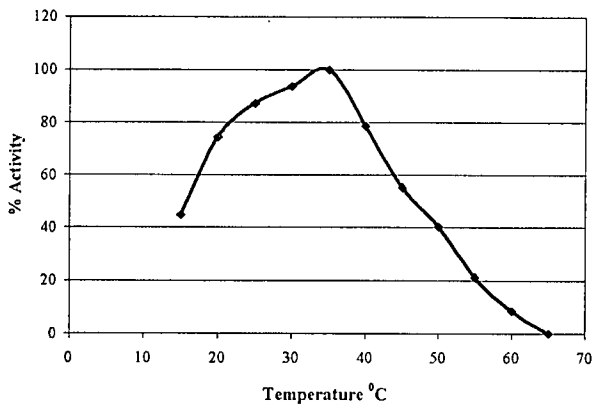


Figure 4.20: Temperature optimum of the purified FAOD.

4.2.4.3 pH stability

Figure 4.21 shows the results of pH stability studies of the purified FAOD enzyme. The enzyme remained fairly stable at pH 7.5 to pH 9.5. Shifting the pH outside this range

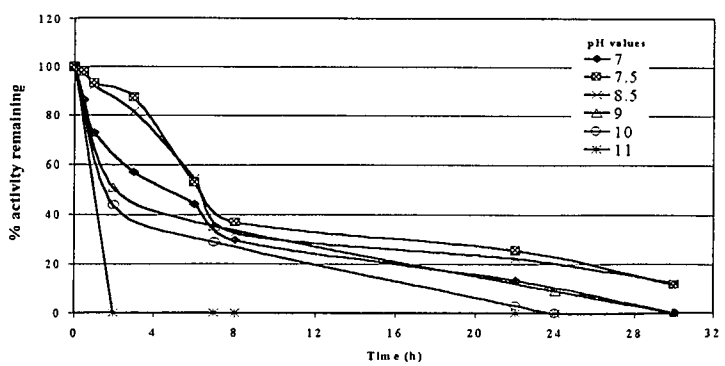


Figure 4.21 FAOD pH stability at 20^{0}C.

resulted in loss of activity with a very rapid loss at values below pH 7.5 whereas a gradual loss of activity was observed in the alkaline pH range.

4.2.4.4 Thermostability

Results of FAOD thermostability studies at temperatures 20^0C to 60^0C are shown in figure 4.22. Approximate half-lives of the enzyme at different temperatures were determined and are shown in Table 4.3. Compared with other enzymes FAOD is not thermostable.

Table 4.3: FAOD half lives at different temperatures.

Temperature ^{0}C	Approximate $T_{0.5}$
20	4h
30	30min
40	10min
50	30sec
60	15sec

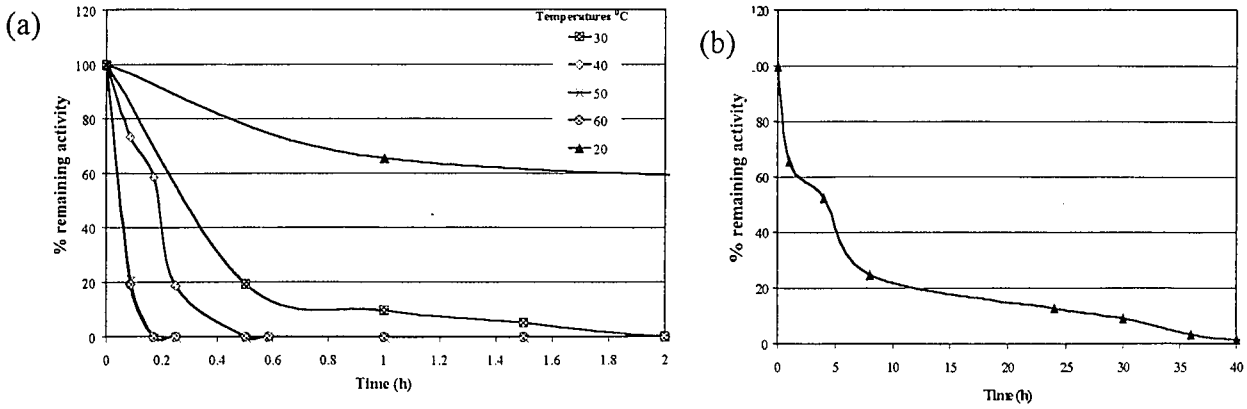


Figure 4.22 Thermostability of FAOD at pH 8.0 and at (a) different temperatures and (b) 20^0C for clarity.

4.2.4.5 Substrate specificity of FAOD

Determination of substrate specificity of FAOD was done using enzyme obtained from the Hexyl agarose 4XL column. The elution profile (Fig. 4.16(c) shows a minor FAOD active peak (tubes 120-150) and a major peak (tubes 200-230). Samples obtained from these two peaks were used for determination of substrate specificity of FAOD. The two peaks are referred to in this section as FAOD low activity peak and FAOD high activity peak respectively.

According to Fig. 4.23 FAOD exhibits a preference for straight chain alcohols ranging from C_9 to C_{12}.

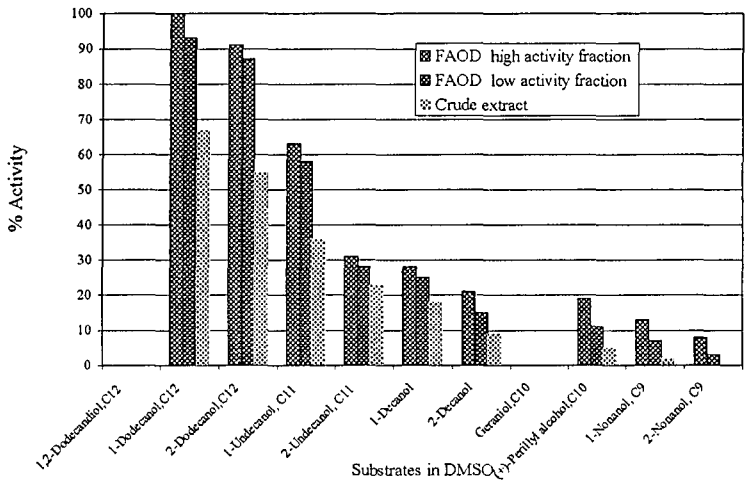


Figure 4.23 Substrate specificity of different peak fractions collected during purification of FAOD.

Straight chain as well as branched or ring-structure alcohols falling outside the C_9 to C_{12} range were not oxidized by the enzyme. The longer chain alcohols (C_{16}, C_{18} and C_{22}) were, however, dissolved in pristane rather than DMSO, because they were insoluble in

DMSO. The effect that this might have had on enzyme activity is not known. It is interesting that although the yeast grew on hexadecane and dodecanol was the best substrate for its FAOD, the FAOD enzyme showed no activity towards 1,2-dodecanediol, 12-hydroxydodecanoic acid, 16-hydroxy-hexadecanoic acid or 1-hexadecanol. The major problem as regards most of these substrates was their insolubility in DMSO. As a result another solvent, 1,4-dioxan, which could dissolve most of the substrates that would not dissolve in DMSO, was used. Before the substrate specificity experiments were carried out the solvent effect of DMSO and 1,4-dioxan on FAOD activity had been determined and compared. The two solvents have no significant effect on FAOD activity (Fig. 4.24).

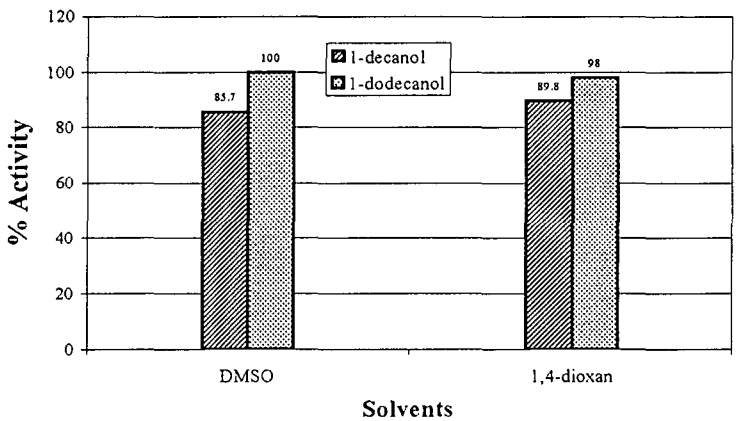


Figure 4.24 The solvent effect of DMSO and 1,4-dioxan on the activity of FAOD. Ammonium sulphate precipitate fraction obtained as described in section 4.2.2.2 was used for this experiment.

However, 1,4-dioxan became the solvent of choice in the following experiments because the majority of substrates used in this experiment were soluble in it. When substrates were dissolved in 1,4-dioxan, there was a small addition to the number of substrates that could be oxidized by the FAOD, namely 16-hydroxyhexadecanoic acid, 12-hydroxydodecanoic acid and 1,2-hexadecanediol (Fig. 4.25). It is obvious that oxidation

of these additional substrates by FAOD was very poor even though these substrates had previously been reported to be suitable substrates for this enzyme (Dickinson and Wadforth, 1992).

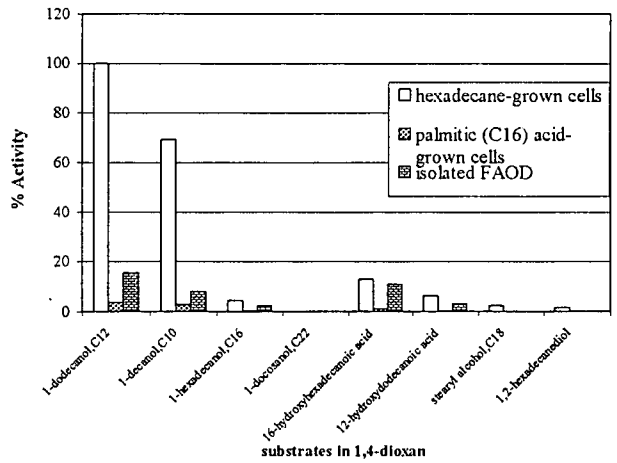


Figure 4.25 FAOD substrate specificity using substrates dissolved in 1,4 dioxan. The FAOD high activity fraction described in section 4.2.4.5 was used for this experiment.

4.3 Comparison of FAOD and FADH cellular levels in *C. tropicalis* OC3 grown on different carbon sources.

Cellular levels of FAOD and FADH in *C. tropicalis* OC3 were determined as described in section 3.2.8 and its two subsections using a crude enzyme preparation which contained both soluble enzymes as well as enzymes solubilized with CHAPS.

According to figure 4.26 the FAOD levels of yeast cells grown on the different hydrocarbons was variable, ranging from negligible (e.g palmitic acid) to very high (e.g oleic acid. It was therefore logical to assume that the yeast must be producing other enzyme(s) responsible for oxidation of the different hydrocarbons the yeast was capable of surviving on, especially when growing on those substrates that were found not to induce FAOD. This other enzyme(s) would perhaps be able to oxidize the rest of the substrates which FAOD was unable to oxidize. The possibility existed that this enzyme could be a fatty alcohol dehydrogenase (FADH) (Ueda and Tanaka, 1990). Production of FADH by *C. tropicalis* OC3 was indeed confirmed. The isolated FAOD enzyme did not have any FADH activity when assayed. This therefore implies that the purification

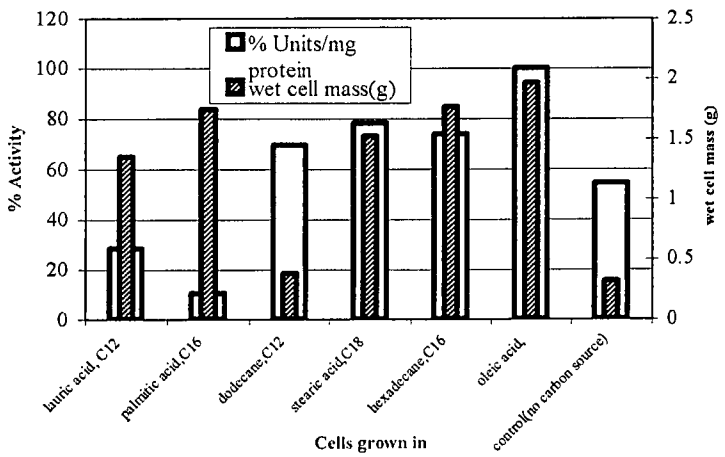


Figure 4.26 Cellular levels of FAOD in *C. tropicalis* OC3 in comparison to wet cell mass of three 20ml cultures that are 42h old. The FAOD active fraction obtained after solubilization of the cell membranes was used for this experiment.

procedure managed to separate the two enzyme activities well.

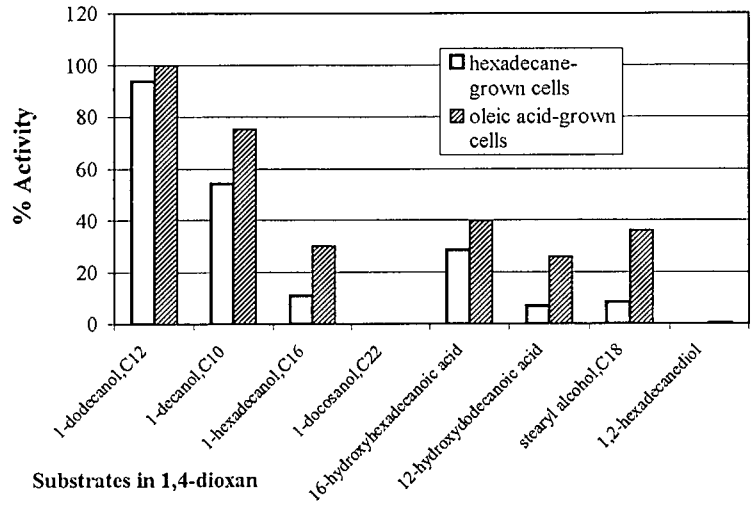


Figure 4.27 Substrate specificity of FADH in *C. tropicalis* OC3 cells grown on hexadecane and oleic acid.

Most of the substrates that FAOD could not oxidize were oxidized by the FADH, even though activities were often still relatively low (Fig. 4.27). Both FAOD and FADH still failed to oxidize the long chain alcohol 1-docosanol (C_{22}). This may be attributed to the insolubility of this substrate in the solvents used. Even though this alcohol could be dissolved in 1,4-dioxan when heated up in a 50^0C water bath, a suspension was formed when the substrate was added to the assay mixture, which was at a lower temperature (30^0C). The cellular levels of FADH and FAOD in the control cells of *C. tropicalis* OC3 are on the average the same. However growth of the yeast cells on hexadecane and oleic acid induced the FAOD cellular levels twice compared to the FADH cellular levels (Figure 4.28). It should also be noted that cellular levels of FAOD are much higher in the small cultures (3 x 20ml) compared to the larger volume cultures (15 x 200ml) used for purification of the FAOD enzyme. The yeast cells seemed to proliferate much better in

small cultures than in the large cultures. The enzyme was similarly induced more in small cultures than in large cultures. Also preparation of solubilized FAOD from smaller cultures is more efficient.

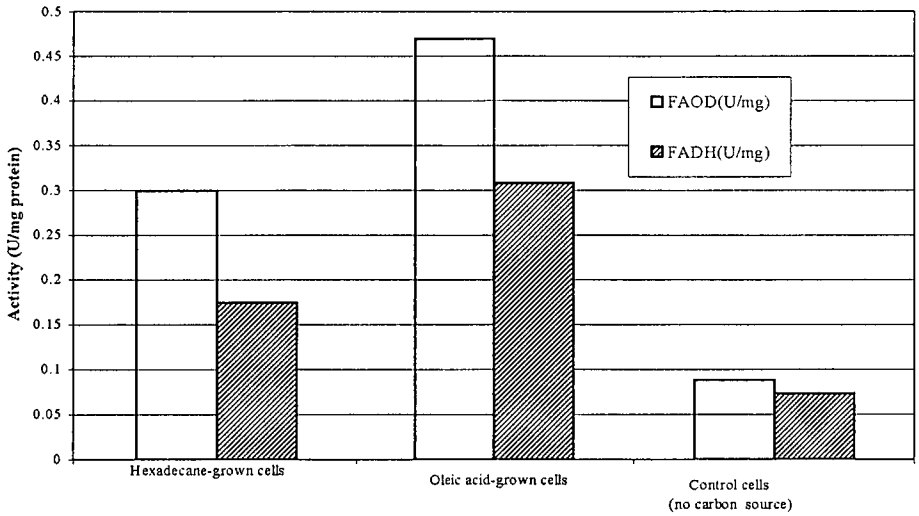


Figure 4.28 A comparison of FAOD and FADH cellular levels in *C. tropicalis* OC3 cells using decanol as the substrate. The FAOD active fraction obtained after solubilization of the cell membranes was used for this experiment.

Note following from external examiners reports: It follows from the external examiners reports that an accurate interpretation of these results is not possible, because the microsomal membrane fraction was not separated from the cytosolic enzymes prior to the solubilization of membrane bound enzymes with CHAPS. Cytosolic alcohol and aldehyde dehydrogenases are present in the yeasts. Activity of these enzymes acting on both the alcohols, as well as formed aldehydes, was probably observed. It is well established that there is no peroxisomal FADH activity (references) in these yeasts, but only a peroxisomal FALDH activity which might also have acted on the formed aldehydes.

CHAPTER FIVE

General Discussion and conclusion

Most intracellular enzymes are known to occur in either one of the three forms – free, fixed in multienzyme complexes, and bound to membrane complexes (Price and Stevens, 1989). The fact that FAOD activity was found to be associated with the membranes of the peroxisomal fraction of *C. tropicalis* OC3 cells therefore did not come as a surprise. This was independent of the method used for cell disruption (i.e. vortexer or bead beater). The enzyme was successfully freed of the membranes by solubilization with the detergent CHAPS (10mM) after which the enzyme was isolated from the membrane fraction. The detergent had no effect upon the enzyme activity.

The elution profiles (fig. 4.16) and purification factors (Table 4.2, Attempts No.13, 14 and 15) indicate that the enzyme might be homogeneous, however, SDS-PAGE patterns obtained are a source of concern (figure 4.17). According to the SDS-PAGE patterns the single activity/protein peak seen from the elution profile does not appear to be homogeneous. Many small bands could still be seen above and below the major protein band. These small bands could be a result of fragmentation of the protein by proteases, either resident in the cell or as an artefact *in vitro*. Proteolytic degradation can also result from enzyme aging and turnover *in vivo*. While the protease inhibitor PMSF has been used and shown to work perfectly well, a mixture of various protease inhibitors will have to be tried in order to be able to completely exclude the proteolytic degradation as the cause of several bands being seen on SDS-PAGE.

One other factor to consider is the very real possibility that the FAOD enzyme may be a member of an enzyme complex, which consists of a number of different subunits making up the complex. It is the dissociation of the complex during the isolation procedure which could have resulted in the evident rapid loss of activity of this enzyme. The appearance of several bands on SDS-PAGE could well represent the different components of the complex. However, the results of size-exclusion chromatography show that our enzyme is composed of only one unit.

A number of multienzyme complexes have been characterized and some have been extensively studied (e.g. the α–ketoacid dehydrogenases and fatty acid synthatase) (de Gruyter, 1983; Royer, 1982). Fortunately the multienzyme complexes that have been most studied are probably amongst the more stable ones, but there may exist others in which the associations are weak and thus more difficult to detect as multienzyme complexes, including the FAOD enzyme under debate.

The isolation and characterization of a multienzyme protein presents more technical problems than a single enzyme as was evident from previously studied complexes (Price and Stevens, 1989). It has been found that different isolation procedures yield complexes of slightly different compositions, some dissociation may occur during isolation and in the intact cell complexes of slightly different composition may exist. Indeed in our various isolation trials of this FAOD enzyme different procedures gave SDS-PAGE patterns that were not comparable at all. It is difficult to determine the M_r of a large complex with sufficient accuracy and thus a small percentage error can significantly

affect the proposed number of subunits of the complex. Evidence for the existence of a multienzyme complex usually appears as two enzyme activities, usually from the same metabolic pathway, that copurify. This is further confirmed if the ratio of the two enzyme activities remains constant during the isolation procedure. The isolated FAOD was assayed for FADH activity only which was negative. There may be other enzyme activities present and these are some of the things that we need to look at in order to confirm if our FAOD enzyme does exist as a multienzyme complex.

The apparent molecular mass, pH and temperature activity profiles of the isolated FAOD enzyme are very similar to those of FAOD's isolated from *Y. lipolytica* (Kemp *et al.*, 1990), *C. tropicalis* (Dickinson and Wadforth, 1992) and *C. bombicola* (Hommel and Ratledge, 1990). The temperature and pH optima values are also very similar to those described for some methanol oxidases (Table 1.1). The thermolabile and photosensitive nature of this FAOD is also not a new phenomenon. Most FAOD's isolated from the majority of alkane-utilizing yeasts have been reported to be both thermolabile and photosensitive to some degree (Kemp *et al.*, 1988, 1990; Hommel and Ratledge, 1990). The degree of lability depends on the organism from which the FAOD was isolated. Due to thermolability and photosensitivity, the storage stability of our enzyme was rapidly reduced by repeated freezing and thawing.

With regard to its substrate specificity the *C. tropicalis* OC3 FAOD is comparable to other FAOD's from alkane–degrading yeasts such as *C. bombicola* (Hommel and Ratledge, 1990), *C. tropicalis* described by Kemp *et al.* (1988) and Dickinson and

Wadforth, (1992). The substrate specificity of our FAOD is, however, slightly narrower, as only primary and secondary alcohols ranging from C_9 to C_{12} were oxidized. Most importantly is, however, the fact that maximal activity was obtained with 1-dodecanol. This has also been reported for FAOD isolated by Dickinson and Wadforth, (1992). It is quite a striking feature that this enzyme is capable of oxidizing 2-alkanols even though these compounds are not intermediates of the catabolic pathway in yeasts. This same property was observed in the FAOD isolated from *C. maltosa* (Mauersberger *et al.*, 1992). Although stereoselectivity in the oxidation of secondary alcohols by our enzyme was not determined it is quite likely that the enzyme could resemble that of *C. maltosa* which exhibits a high stereoselectivity for these compounds. This property could well make this enzyme suitable for the preparation of enantiomerically pure alcohols.

A comparison of the isolated FAOD with the alcohol oxidase of *C. cloacae* isolated by Vanhanen *et al.* (2000) shows a close similarity between the two enzymes with respect to M_r, pH and temperature optima. The alcohol oxidase of the *C. cloacae* and the one from *C. tropicalis* isolated by Dickinson and Wadforth, (1992) have been reported to have a wide substrate specificity, oxidizing both straight chain alcohols and ω-hydroxy fatty acids. Moreover the alcohol oxidase of *C. cloacae* is strongly induced by growth of this yeast on oleic acid. In addition to alkanes the *C. cloacae* yeast can utilize a wide range of fatty acids as sole sources of carbon. As a result Vanhanen *et al.* (2000) named the alcohol oxidase enzyme induced by growth of this yeast on fatty acids a long chain (LC)-fatty acid alcohol oxidase. Our FAOD enzyme was also induced by growth of the *C. tropicalis* OC3 yeast on n-alkanes and fatty acids. However, the enzyme poorly oxidized

ω-hydroxy fatty acids and would not oxidize diols and long chain alcohols (C_{16}, C_{18} and C_{22}). This behaviour is very similar to that of the FAOD of the fungus *Aspergillus flavipes* (Savitha and Ratledge, 1991) which, though it oxidized long chain alcohols, would not oxidize α,ω-diols, hydroxy fatty acids and secondary alcohols. This kind of behaviour among FAOD's may imply that different FAOD genes code for FAOD's of slightly varying substrate specificity or cellular location. This therefore explains why our isolated FAOD though similar in many ways to the FAOD's described by Dickinson and Wadforth, (1992) and Vanhanen *et al.* (2000) behaves differently in some aspects. The conserved FAOD sequences homologous to some sequences in *Arabidopsis* and *Mycobacterium* found by Vanhanen *et al.* (2000) may explain the similarities observed in alcohol oxidases of yeasts, plants and bacteria. This further suggests the presence of yet unidentified family of long chain alcohol oxidases linking eukaryotes to prokaryotes.

It is obvious from the previous discussion that a lot of information is still unknown about the FAOD enzyme, and as such the protein is still open to investigation. The data collected from this study thus serves as a starting point for further characterization of this enzyme. Characterization includes, *inter alia*, elucidation of whether the enzyme exists in a complex or not, the prosthetic group responsible for the light sensitivity nature of the protein and the molecular properties of the FAOD gene(s). Analysis of the FAOD gene structure will provide valuable information regarding homology to related enzymes and control of expression and thus greatly facilitate tailoring for industrial applications.

In conclusion one can say that FAOD and some other enzymes involved in the early stages of the alkane-degradation pathway remain a fascinating group of enzymes that are genetically controlled by some several length-specific alkane-uptake systems enabling them to selectively break down alkanes of all chain lengths possibly found in nature. Revelation of the control engines of this complicated system would be a break through in the world of genetic manipulation of alkane-degrading yeasts, especially for the petrochemical industry where huge quantities of these long chain alkanes have to be disposed of instead of becoming a utilizable and profitable product.

Future Research

Vanhanen *et al.* (2000) have obtained a full-length coding sequence of a long-chain (LC) fatty acid alcohol oxidase from *C. cloacae* and have already used this sequence information to successfully clone and isolate a fatty acid alcohol oxidase from *C. tropicalis* yeast strain. It would therefore be logical and easier to use fatty alcohol oxidase sequences of the two yeasts to design primers (or a DNA probe) and screen a cDNA library of the *C. tropicalis* OC3 for the FAOD gene(s). When the gene has been cloned and sequenced it can then be characterized and manipulated as desired. If the gene is successfully overexpressed it can also be used for isolation of the enzyme and this one believes would be far easier than starting at purification of the enzyme as is in our case.

The concurrent occurrence of both FAOD and FADH in our *C. tropicalis* yeast has been confirmed. It is therefore obvious that if the yeast system responsible for alkane-degadation is to be effectively manipulated as desired genes coding for FAOD and FADH will have to be targeted both. Otherwise the FADH gene may well make up for the defects resulting from manipulation of FAOD gene. If this becomes the case then these defects may be very much insignificant and/or be not observable at all.

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Summary

Fatty alcohol oxidases (EC 3.1.1.13) (FAOD) are enzymes induced by growth of yeast on long chain alkanes. These enzymes catalyse the oxidation of long chain (fatty) alcohols to fatty aldehydes. Many products of the alkane-assimilation pathway such as dicarboxylic acids, long chain alcohols and aldehydes are of industrial importance in the production of detergents, lubricants, surfactants and cosmetics. Currently, production of these products involve extraction from natural sources or synthesis from petrochemicals, but neither method is satisfactory. The potential of synthesizing such value-added products using alkane degrading yeasts is thus being investigated. Knowledge of the genes coding for enzymes responsible for production of these products by yeasts would facilitate genetic manipulation of the yeasts, so that it becomes possible to accumulate products of the alkane-assimilation pathway.

Isolation of fatty alcohol oxidase from *C. tropicalis* OC3 was carried out by first harvesting and disrupting the cells to release the enzyme. The cell-free crude extract was subjected to differential centrifugation to obtain the FAOD-containing peroxisomal fraction. The peroxisomal fraction was solubilized with a detergent, CHAPS, to release the enzyme from the membranes. Isolation of the FAOD enzyme was achieved using ammonium sulphate fractionation followed by hydrophobic interaction chromatography on a Hexyl agarose 4XL column. Other chromatographic columns which were tried and found to be unsuitable for purification of this enzyme include the anion-exchangers QAE-Sephadex and DEAE-Toyopearl 650M, as well as affinity chromatography MIMETIC™ dye ligands, Blue 1 and Yellow 2. The MIMETIC™ Blue 2 column seems to be the ideal

column for purification of the FAOD enzyme, however very unfortunately, the hunt for better columns went on for too long and in the end there was no time to try the Mimetic Blue 2 column again.

The final purification protocol for FAOD from *C. tropicalis* OC3 resulted in a 73-fold purification, a specific activity of 1.17U/mg and a final yield of about 48%. One major band with an approximate molecular mass of 75 000 to 80 000 was obtained after SDS-PAGE. The purified enzyme had an optimum activity at pH 9.5 and 35°C. The pH stability of the enzyme was found to be in the range pH 7.5 to 10 although the enzyme retained only about 60% activity at pH 7.0. The enzyme was not stable at temperatures above 20°C, exhibiting an approximate half-life of 4 hours at 20°C and only 30 minutes at 30°C. Substrate specificity studies showed that this FAOD prefers primary and secondary alcohols in the range C_9 to C_{12}. Even though it has been reported (Dickinson and Wadforth, 1992) that long-chain alkane-diols and ω-hydroxy acids are substrates for this group of enzymes we found that 1,2-hexdecanediol, 16-hydroxydodecanoic acid and 12-hydroxydodecanoic acid were poorly oxidized by this FAOD. An anomally was that the cells from which the enzyme was isolated were grown on hexadecane but the enzyme showed very low activity for hexadecan-1-ol. We found that in addition to FAOD the yeast cells also produced a fatty alcohol dehydrogenase (FADH) enzyme. This enzyme might enable the yeast to grow on a variety of hydrocarbon sources even when its FAOD cellular levels are low.

Even though SDS-PAGE results showed that the FAOD protein was not homogeneous, we concluded from the nature of the elution profiles and the specific activity values that the isolated FAOD enzyme is probably pure enough to submit for amino acid sequencing. However, Vanhanen *et al.* (2000) recently published three gene sequences of what they call long-chain fatty acid alcohol oxidases from *C. cloacae* and *C. tropicalis*. It would now probably be easier using this information to locate the FAOD gene(s) of our *C. tropicalis* OC3 strain.

Opsomming

Langketting alkohol oksidases (EC 3.1.1.13) (FAOD) is ensieme wat geinduseer word tydens groei van giste op lang ketting alkane. Hierdie ensieme kataliseer die oksidasie van lang ketting alkohole na die ooreenkomstige aldehiede. Baie produkte van die alkaan assimileringsweg soos dikarboksielsure, lang ketting alkohole en aldehiede is van industriele belang vir die produksie van detergente, smeermiddels, surfaktante en kosmetiese preparate. Tans behels die produksie van hierdie verbindings ekstraksie uit natuurlike bronne of sintese vanaf petrochemiese substrate, maar nie een van bogenoemde metodes werk bevredigend nie. Die moontlikheid om alkaan benuttende giste te gebruik vir die produksie van hierdie waardevolle produkte word dus ondersoek.. Kennis van die gene wat kodeer vir die ensieme verantwoordelik vir hierdie reaksies in giste sal dit moontlik maak om die giste geneties te manipuleer, sodat hulle van die waardevolle tussenprodukte kan ophoop.

Om die lang ketting alkohol oksidase van *C. tropicalis* OC3 te isoleer is die selle eers geoes en gebreek. Die selvrye ru-ekstrak is onderwerp aan differensiele sentrifugasie om die oksidase bevattende peroksisoom fraksie te verkry. Die peroksisoom fraksie is gesolubiliseer met 'n detergent, CHAPS, om die ensiem vry te stel uit die membrane. Die alkohol oksidase ensiem is geisoleer deur ammonium sulfaat fraksionering gevolg deur hidrofobiese interaksie chromatografie op 'n Heksiel agarose 4XL kolom. Ander chromatografiese kolomme wat probeer is, maar nie geskik was vir die suiwering van die ensiem nie sluit in die anioon-uitruilers QAE-Sephadex en DEAE-Toyopearl 650M,

asook affiniteitsmatrikse met MIMETIC^{TM} kleurstof ligande, Blue 1, Blue 2 and Yellow 2.

Met die finale suiweringsprotokol kon die langketting alkohol oksidase van *C. tropicalis* OC3 geisoleer word met 'n suiweringsfaktor van 73, 'n spesifieke aktiwiteit van 1.17 U/mg en 'n finale opbrengs van 48%. Een hoof band met 'n molekulere massa tussen 70 000 en 75 000 is verkry na SDS-PAGE. Die gesuiwerde ensiem het optimale aktiwiteit gehad by pH 9.5 en 35°C. Die pH stabiliteit van die ensiem was in die gebied pH 7.5 tot 10 alhoewel die ensiem slegs 60% van optimale aktiwiteit behou het by pH 7.0. Die ensiem was nie stabiel by temperature bo 20°C nie met 'n halfleeftyd van ongeveer 4 ure by 20°C en slegs 30 minute by 30°C. Substraat spesifisiteit studies het gewys dat hierdie ensiem primere en sekondere alkohole met ketting lengtes C_9 tot C_{12} verkies. Alhoewel dit geraporteer is (Dickinson and Wadforth, 1992) dat lang ketting alkaan diole en ω-hydroksiesure substrate is vir hierdie groep ensieme, het ons gevind dat 1,2-heksaandiol, 16-hidroksieheksadekanoësuur and 12-hidroksiedodekanoësuur baie swak deur hierdie ensiem geoksideer word. Veral vreemd is die waarneming dat die ensiem geisoleer is vanaf selle wat opgegroei was op heksadekaan as substraat, maar heksadekan-1-ol baie swak okdsideer. Ons het gevind dat die giste behalwe die alkohol oksidase ook 'n lang ketting alkohol dehidrogenase (FADH) ensiem produseer. Laasgenoemde ensiem mag die gis in staat stel om op 'n verskeidenheid koolwaterstowwe te groei selfs wanneer die alkohol oksidase aktiwiteit laag is.

Alhoewel SDS-PAGE getoon het dat die oksidase protein nie homogeen was nie, het ons op grond van die elueringsprofiele en die finale spesifieke aktiwiteit besluit dat die geisoleerde oksidase ensiem waarskynlik skoon genoeg was om 'n aminosuurvolgordebepaling te laat doen . Vanhanen *et al.* (2000) het egter onlangs die basispaar volgordes gepubliseer van die gene wat kodeer vir drie sogenaamde langketting vetsuur alkohol oksidases in *C. cloacae* en *C. tropicalis.* Dit sal nou waarskynlik makliker wees om hierdie inligting te gebruik om die geen wat kodeer vir die langketting alkohol oksidase in ons *C. tropicalis* OC3 stam op te spoor.